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SECTION MEETINGS

CLEVELAND, O. Western Reserve University	March 21, 1960
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Effect of Beta-Sitosterol on Regression of Hyperlipemia and Increased Plasma Coagulability in the Chicken.* (25769)

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As an etiological factor in atherogenesis, lipids have received a great deal of study. However, as an etiological agent in the formation of thrombi, they have only recently become of interest to investigators. Within this decade, Fullerton *et al.*(1) correlated high blood lipid levels with increased blood coagulability in the human. This past year, reports were published on production of experimental infarcts in animals fed a lipemia-inducing diet(2,3,4). This report deals with production of an experimental hyperlipemia and increased plasma coagulability in the chicken and its subsequent reversal by feeding beta-sitosterol.

Methods. Six-week-old cockerels were housed in a broiler battery. A broiler ration and water were allowed *ad libitum* during a one-week acclimatization period. Plasma lipid levels and plasma coagulation times were then determined on randomly selected birds. The cockerels were then randomly divided into 5 diet groups: A, broiler ration (7% fat, 24% protein, 3% crude fiber); B, broiler ration + 5% cottonseed oil, (basal ration); C, basal

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ration + 1% cholesterol; D, basal ration + 1% cholesterol + 4% sitosterol; E, basal ration + 4% sitosterol; C-D, one-half group C placed on diet D at 4-week test period. Each bird was bled at 2, 4, and 6-week intervals after being placed on the diets. At 4 weeks, Group C was divided into 2 equal groups. One group was placed on diet C-D to determine whether regression of hyperlipemia and hypercoagulability would occur; whereas the other group remained on diet C. Blood was drawn by cardiac puncture and was citrated in the syringe. An aliquot of the plasma was extracted with 1:1 alcohol-acetone for lipid analysis; plasma coagulation, or "Stypven," time was determined according to Page and Russell(5) on the remaining plasma. When the experiment was terminated, liver and aorta were removed for determination of total cholesterol and total lipid. Total cholesterol was determined by the method of Sperry and Webb(6); total lipids were determined gravimetrically after treating the alcohol-acetone extract as outlined by Folch(7); lipid phosphorus was estimated on the residue from the total lipid analysis by the method of Fiske and Subba-Row(8).

Results. Data for blood lipids are presented in Table I. Irrespective of dietary treatment and time, a marked increase in total lipids and cholesterol levels was found in Group C during the experimental period. Group D, which received 4% sitosterol in addition to the Group C diet, did not exhibit a hyperlipemia. At 6 weeks, Group C-D, which was transferred from diet C to diet D at the end of the 4-week test period, had normal lipid and cholesterol levels. This represented a regression of hyperlipemia of the birds derived from Group C. In Group C, an increased lipid phosphorus was found at 4 and 6 weeks, whereas Group C-D had lipid phosphorus levels equal to those of the control group at 6 weeks.

TABLE I. Effect of Dietary Additions on Plasma Lipid Concentration* of Chickens.

Diet group	Time	Total lipids	Total chol.	Lipid-P
Control	0	279	82	4.8
A	4 wk	215	89	3.4
B		239	90	3.2
C		985	385	4.6
D		307	111	3.3
E		325	101	2.8
A	6 wk	252	83	3.3
B		300	88	3.9
C		983	534	5.8
D		377	102	4.2
E		340	85	4.7
C-D		338	113	4.2

* Data expressed as mg/100 cc plasma.

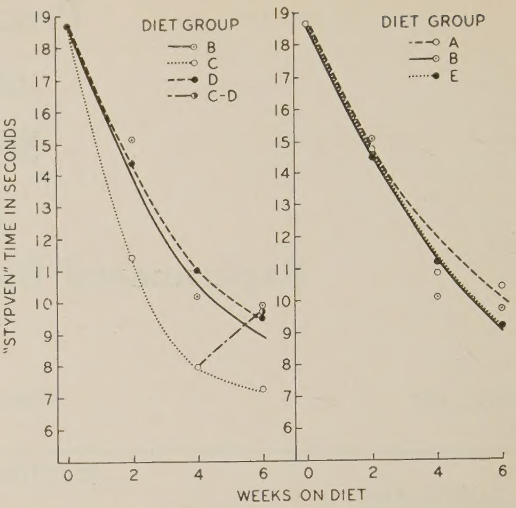

FIG. 1. Effect of diet on "Stypven" in chickens.

Plasma coagulation data, or "Stypven" times, are presented in Fig. 1. Throughout the course of experiment, a decrease in "Stypven" time was found in all groups. However, as may be ascertained from the curves, rate of decrease was the same in all control groups. Group C, which exhibited a marked increase in blood lipids, had a significantly greater rate of decrease of "Stypven" time during the experimental period. Group D, which was receiving diet C with an addition of 4% sitosterol, had "Stypven" times commensurate with those of Group B on a basal ration. Group C-D, which was placed on diet D after 4 weeks on diet C, had "Stypven" times equal to the control groups at 6 weeks. The effects of dietary additions on aorta and liver total lipids and cholesterol are presented in Table II. A marked increase of total lipids and cholesterol in these tissues was observed only in Group C. When 4% sitosterol was added to the diet and fed during the entire experimental period, an increase in total lipids and cholesterol of these tissues was prevented in Group D. Group C-D, which was transferred

TABLE II. Effect of Dietary Additions on Aorta and Liver Lipid Concentrations.

Diet group	Aorta* Total lipids	Aorta* Total chol.	Liver† Total lipids	Liver† Total chol.
A	117	3.5	28.5	3.5
B	108	4.2	32.0	3.2
C	165	7.4	77.9	35.0
D	126	4.1	30.2	3.5
E	133	3.8	28.9	3.1
C-D	113	4.9	29.8	3.7

* Data expressed as mg/g dry tissue.
† " " " " wet " .

to diet D at the end of the 4-week test period, was found to have normal lipid and cholesterol levels in liver and aorta.

Discussion. In the chicken, development of a hyperlipemia, and a concomitant hypercholesteremia, appears to be dependent upon presence of cholesterol in the diet. Both Peterson(9,10) and Pollack(11) have reported independently that a hyperlipemia was not observed when a cholesterol-free diet, or a diet containing cholesterol and sitosterol, was fed to chickens or rabbits. Regression of cholesterol-induced hyperlipemia and atheroma was observed when sitosterol was added to the diet containing cholesterol(12). Addition of sitosterol to the diet presumably inhibits cholesterol absorption. Thus, normal lipid levels are obtained and, depending upon the experimental situation, atheromas are either not formed or existing ones regress. Fullerton *et al.*(1) observed a decrease in clotting time of human whole blood, and of plasma in the presence of Russell's viper venom, after ingestion of a fatty meal. Tilden and Shipley (13) have reported similar observations in dogs. Comparable results were obtained by Shoulders *et al.*(14) and Swank(15) upon intravenous administration of lipid emulsions to dogs; Swank(15) produced similar results in rabbits. Among the agents suspected of shortening plasma clotting time are chylomicrons, certain fats, and certain phospholipids. McDonald and Fullerton(16) observed that decreased clotting time was obtained in humans with restricted physical activity; a fat meal decreased this time still further. This lack of physical activity may be a contributing factor in the decreased "Stypven" time observed in the control chickens of this report.

It is generally accepted that atherosclerosis and/or thrombosis are pathological states leading to tissue infarcts. Interrelationships of those pathological states are unknown. Whether one predisposes to the other, and to what extent each acts independently of the other to cause an infarct, remains to be investigated. From evidence accumulated to date, chronically elevated blood lipids are factors predisposing to atherosclerosis and thrombosis. Indeed, O'Neal *et al.*(2), Thomas and Hartroft(3), and Wilgram(4) have demonstrated that experimental infarcts may be produced in rats with a lipemia and atheroma-inducing diet, *i.e.*, a high fat diet with cholesterol. It is unknown whether hypercholesteremia is a concomitant, or causative agent, of hyperlipemia. It is interesting to note, however, that experimental production, or regression, of atheromas and changes in plasma coagulation times may be controlled in young cockerels with beta-sitosterol, an agent which specifically inhibits cholesterol absorption.

Summary. 1. A cholesterol-induced hyperlipemia was fully counteracted (reduced to normal levels) by feeding beta-sitosterol. 2. The decreased plasma clotting times associated with a hyperlipemia returned to control values when a normal lipid level was attained. 3. An inverse relationship was demonstrated between plasma clotting time in presence of Russell's viper venom and degree of lipemia; *i.e.*, a hyperlipemia resulted in decreased plasma clotting time, and vice versa. 4. Addition of 4% beta-sitosterol to the diet prevented or reversed an increased lipid and cholesterol concentration in liver and aorta.

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Parotoid Secretions of *Bufo blombergi* and *B. peltocephalus*. (25770)

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Bufo blombergi, a giant toad of Colombia, was recently discovered by Rolf Blomberg(1, 2). Each toad weighs one kg or more. *B. peltocephalus* (or *peltacephalus*(3)), a toad well known in Cuba, is comparable in size to *B. marinus*. In contrast with other species, the longitudinal axis of its parotoid glands is perpendicular to the body axis(4). Both *B. blombergi* and *B. peltocephalus* were in the collections of the Dept. of Amphibians and Reptiles of New York Zoological Park. Since no chemical and pharmacological information is available about their parotoid secretions, it was felt desirable to make a preliminary examination. By permission of Dr. James A. Oliver, Director of the New York Zoological Park, one of us (K.K.C.) expressed the parotoid secretions from 2 animals of each species into 2 glass vessels, according to procedure previously described(5). The light yellow secretion of the Colombian toad was milky, slimy, and quickly developed a consistency of chewing gum. It had no special odor. Secretion of the Cuban toad was light gray, creamy, and had a slight scent. Both secretions were air-dried and then desiccator-dried. The material from *B. blombergi*, total 2.156 g, remained light yellow. It was very difficult to pulverize in a mortar because of stickiness. Chunks were unavoidable. No other toad secretion has shown this property. The venom of *B. peltocephalus*, total weight 0.237 g, turned black on drying, and could easily be ground into a fine powder. Sneezing, a bitter taste, and numbness of tongue were noticed during grinding. Various extracts were prepared for detection of catecholamines, indolethylamine derivatives, cholesterol, and cardiotonic substances.

Results. 1. *Catecholamines.* Ten-mg samples were repeatedly extracted with 0.1 N HCl, filtered, and diluted to 10 ml. When ammoniacal alcohol was layered over extracts of both toad secretions, a rose-red color, typical of catecholamines, developed at the junction(6). Intravenous injection of these solutions in pithed cats was followed by a rapid rise of carotid blood pressure with prompt recovery, the pressor action of *B. peltocephalus* being greater than that of *B. blombergi*. Quantitative comparisons were attempted by paper chromatography(7,8). The acid extracts were spotted on Whatman No. 1 paper and developed with water-saturated phenol by ascending chromatography in an atmosphere equilibrated with concentrated HCl and phenol. Air-dried tapes were sprayed with potassium ferricyanide in phosphate buffer, pH 7.8, to make the spots visible. Estimations of epinephrine content were made by comparing spot intensities with those produced by known quantities of epinephrine. Epinephrine concentrations in dried secretions of *B. peltocephalus* and *B. blombergi* were 10% and 2% respectively. Best estimates of *nor*-epinephrine in *B. peltocephalus* were 0.5 to 1%. The existence of this base in the secretion of *B. blombergi* could not be detected by the

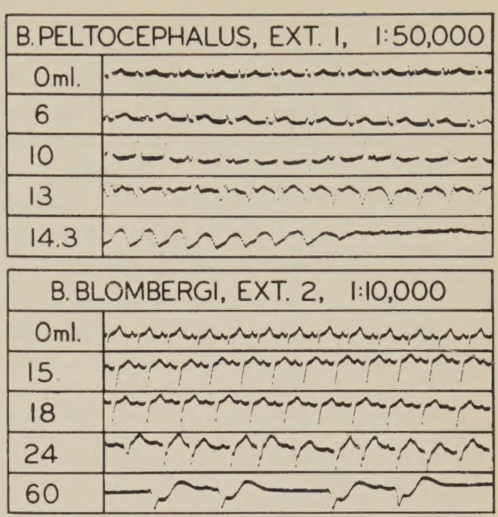

FIG. 1. Action of cardiotonic substances on electrocardiogram. Both cats under ether anesthesia. Electrocardiograms taken continuously from Lead II. Rate of intrav. inj.: 1 ml/min. Note difference of bottom tracings: cat receiving extract from Cuban toad died of ventricular fibrillation, whereas the one receiving that of Colombian toad showed slow ventricular beats almost indefinitely.

method employed. Use of a spectrofluorometer would be necessary to establish any trace of *nor*-epinephrine in this species. Presence of *nor*-epinephrine in parotoid secretions of Chinese and marine toads has been independently proved(9,10). The high content of catecholamines in *B. peltocephalus* would explain the black color after drying due to oxidation. The presence of epinephrine in *B. blombergi* corrects an earlier conclusion based on a less sensitive method, that no epinephrine was present(11). 2. *Indolethylamines.* Samples of 10 mg of dried secretions of both species were crushed in 2 ml of 95% ethanol and allowed to stand at room temperature for 96 hours. Water was added to each extract, followed by filtration and washing through sintered glass to give a volume of 15 ml. The extracts when treated with a *p*-dimethylaminobenzaldehyde test solution U.S.P.(12) gave a blue-green color that was more intense with *B. peltocephalus.* A similar color was observed with serotonin. When the color reagent was modified by substitution of sodium nitrite for ferric chloride(13), a deep purple was produced, which turned red upon standing. Serotonin again duplicated the results. The color reaction does not necessarily indicate presence of serotonin in the secretions of the 2 toads, but it may be evidence of its analogs, especially since in other toads derivatives of 5-hydroxyindolethylamine have been identified(14). 3. *Cholesterol.* A 5-mg sample of each dried toad poison was treated with potassium hydroxide in ethanol, and incubated for 30 minutes at 37°C(15). The whole was extracted with petroleum ether and the supernatant layer was evaporated to dryness. The residue was dissolved in 0.25 ml of chloroform and an aliquot of 0.05 ml (equivalent to 1 mg of the secretion) was spotted on paraffin-treated paper for chromatography, by means of a system of cellosolve, *n*-propanol, methanol and water(16). Cholesterol was found to be present in each of the 2 secretions, decidedly more in *B. blombergi* than in *B. peltocephalus.* 4. *Digitalis-like substances.* A 50-mg sample of each secretion was extracted with methanol for one week under frequent agitation. The extract was filtered and reduced to 10 ml. This was called extract No. 1. For *B. blombergi* a second extract, called extract No. 2, had to be made with twice the quantity of secretion because of its low potency. When extract No. 1 of *B. peltocephalus* was diluted to 1:50,000 and infused at the rate of 1 ml/min into the femoral vein of an etherized cat, characteristic electrocardiographic changes were observed (Fig. 1). The cat died of ventricular fibrillation. Two other cats succumbed in the same manner. With extract No. 2 of *B. blombergi,* a 1:10,000 dilution produced slowing of sinus rhythm, P-R prolongation and A-V dissociation, but failed to cause cardiac stoppage with as much as 60 ml (Fig. 1). Two additional animals were studied in the same manner and similar results were obtained. This may indicate low potency of cardiotonic substances. Convulsant movements as observed resembled those produced by 5-anhydro-periplogenone(17).

In frogs, 0.4 to 1.1 ml of undiluted extract No. 1 from either toad, injected into the ventral lymph sac, did not induce systolic ventricular standstill. This could have been due to lack of absorption of the active substances.

Summary. Dried parotoid secretions of *B.*

peltocephalus and *B. blombergi* have been studied for presence of substances known to occur in secretions of other toads. Catecholamines and indolethylamine derivatives were found in both samples, more in *B. peltocephalus* than in *B. blombergi.* Cholesterol was also detected in both species. The digitalis-like substances of *B. peltocephalus* were more potent in cats than those of *B. blombergi.*

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Effect of Thiamine Deficiency on Glutathione Contents of Erythrocytes and Tissues in the Rat. (25771)

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Thiamine-deficient rats excrete a significant amount of methyl glyoxal which is not found in the urine of treated ones. This phenomenon is probably attributable to reduction of glyoxlase activity in liver(1). The mechanism for the decrease of this enzymatic action is, however, not clear. Since glutathione is known as an essential co-factor for glyoxlase activity(2), it becomes important to ascertain whether reduction of glyoxlase activity is related to changes of glutathione content of thiamine-deficient animals. Experiments, therefore, were undertaken to determine the influence of thiamine deficiency on glutathione levels of erythrocytes and tissues in the rat.

Materials and methods. Twenty-five-day-old male rats weighing from 32 to 41 g were used for this study. They were randomly distributed into 2 groups of 6 each. One group received the basal diet supplemented with all known vitamins, except thiamine. The percentage composition of the basal diet has been shown elsewhere(3). The other group was kept on the same basal diet supplemented with thiamine HCl (0.22 mg/100 g basal diet). All animals were housed individually in screen-bottom cages and were offered drinking water *ad libitum.* The feeding was continued for 4 weeks in the first experiment and 8 weeks in the second experiment. Animals were fasted overnight before being sacrificed. Heparinized blood samples were drawn by direct cardiac puncture. A small portion of liver (approximately 0.5 g) was quickly excised in the first experiment; and, in addition, liver tissue, one left kidney and whole heart were removed from each rat in the second experiment. Glutathione content of blood and tissues were measured by the nitroprusside test as described previously(4).

TABLE I. Effect of Thiamine Deficiency on Growth and on Erythrocyte and Liver Glutathione Content.

Dietary treatment	Final body wt, g	Erythrocyte, μM/100 ml R.B.C.	Liver, μM/100 g wet tissue
Thiamine-deficient	89 ± 9.1 (6)*	176 ± 13.1	715 ± 31.9
Thiamine-treated	144 ± 3.3 (6)	321 ± 21.9	389 ± 59.7
t value	9.78	6.24	5.76

* Mean and stand. error of mean. No. in parenthesis denotes No. of rats used.

Results. The effect of thiamine deficiency on erythrocyte and liver glutathione contents and final body weight is presented in Table I. It indicates that thiamine deficiency results in an alteration of glutathione metabolism. Mean glutathione value in erythrocytes of thiamine-deficient rats is approximately 54% of that of animals raised on a thiamine-supplemented diet. Hepatic glutathione activity of thiamine-depleted rats was 84% higher than that of rats receiving thiamine in the diet.

The second experiment was primarily undertaken to study whether or not other vital organs, besides liver, will have altered glutathione concentration as a result of prolonged feeding of thiamine restricted diet. The results are shown in Table II. Again, glutathione level in erythrocytes was reduced and that in liver was elevated in thiamine-deficient rats. Furthermore, thiamine deficiency results in a 46% decrease of glutathione activity in cardiac muscle, but has no effect on level of glutathione in kidneys.

The finding that concentration of the important regulator of cell metabolism, glutathione, is diminished in erythrocytes under the dietary restriction of thiamine is of importance since glutathione plays a significant role in carbohydrate metabolism. Barron(5) postulated that one of the most important functions of glutathione is to maintain the activity of sulfhydryl enzymes in metabolism. A direct relationship between glutathione and carbohydrate metabolism has been reported by Krimsky and Racker(6) who demonstrated that glutathione is the prosthetic group of glyceraldehyde-3-phosphate dehydrogenase. Thus, it is quite possible that any alteration of glutathione content in the body will consequently entail derangements in carbohydrate metabolism. In view of the involvement of thiamine as thiamine pyrophosphate in pyruvate metabolic pathways, it is of interest to point out that the increase in level of pyruvate and lactic acid in blood of thiamine-deficient rats might be partially due to decreased activity of glutathione in the erythrocytes.

The increase in amount of glutathione in the liver of thiamine-depleted animals suggests that there is little or no correlation with reduction of glyoxlase activity. In this organ, glutathione is broken down and resynthesized (7,8). The present findings do not indicate that thiamine participates either in the degradation process or in the biosynthesis of this tripeptide.

TABLE II. Effect of Thiamine Deficiency on Erythrocyte and Tissue Glutathione Content.

Dietary treatment	Final body wt, g	Erythrocyte, μM/100 ml R.B.C.	μM/100 g wet tissue Liver	Kidneys	Heart
Thiamine-deficient	80 ± 10.5 (6)*	130 ± 10.7	577 ± 12.8	361 ± 25.8	104 ± 11.3
Thiamine-treated	233 ± 20.9 (6)	272 ± 19.7	401 ± 40.6	354 ± 23.5	188 ± 9.5
t value	7.80	7.20	4.92	.27	6.84

* Mean and stand. error of mean. No. in parenthesis denotes No. of rats used.

Summary. Thiamine deficiency results in decrease in concentration of glutathione in erythrocytes and heart, but an increase in level of glutathione in liver tissue. The exact nature of the alteration of glutathione content is not known. The possible interrelationship of thiamine and glutathione in carbohydrate metabolism is discussed.

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Response of Adrenals, Thymus, Spleen and Leucocytes to Shuttle Box and Confinement Stress.* (25772)

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Previously reported studies indicated that exposure of mice to stress-induced emotional disturbance of 2-4 weeks of daily sessions in shuttle box or of physical restraint increase their susceptibility to herpes simplex and Coxsackie B1 viruses(1,2). In contrast, susceptibility to anaphylaxis(3) and to homograft reaction, delayed hypersensitivity(4), decreases after one or more exposures to shuttle box stress. The nature of changes in host-response has not been determined. However, known sensitivity of the pituitary-adrenal, and thymico-lymphatic and blood leucocytic systems to all forms of stress(5), together with the importance of these systems in resistance to infection(6), suggests that they may be related to changes in resistance following stress exposure. Our report describes responses of the above systems in mice exposed to stressors mentioned, as reflected by changes in adrenal, thymus, and spleen weights, and blood leucocytes. These measures were chosen primarily to elucidate mechanisms related to resistance but with the hope that results might also provide other criteria by which stress response could be measured.

Materials and methods. The stressors used, shuttle box and confinement, have been described. The latter stressor, in contrast to shuttle box, tends to minimize activity and avoids shock. Four to 6-week-old female Swiss mice were used(1). Experimental animals were subjected to stress by one of the methods indicated for 6 hours/day, 6 days/week. Control animals were maintained in home cages from which food and water were withdrawn for 6 hours daily. Subgroups of 10 animals and 10 controls were sacrificed after varying intervals of stress exposure, and varying periods of recovery following termination of 28 days exposure to stress. Animals were sacrificed under anesthesia usually within 4 hours after the last stress period. Controls were sacrificed in late morning or early afternoon of days on which stressed animals were killed. Organ weights were determined and expressed as mg/g of mouse weight determined immediately before death. For histological examination, organs were fixed in Bouin's solution, imbedded in paraffin, sectioned and stained with hematoxylin and eosin. Total leucocyte counts were made on tail blood. Differential leucocyte counts were made after May-Gruenwald Giemsa staining. Two hundred leucocytes were counted and separated into the following categories: Polymorphonuclear leucocytes, normal lymphocytes, stress lymphocytes(7), monocytes and eosinophiles.

Results. Shuttle box stress. The data summarized for shuttle box in Table I and in Fig. 1-4 represent composite means from several experiments with the following numbers of animals at intervals indicated: (1) during stress, 20 at 1 d.; 20 at 30 d.; 40 at 21 d. and 110 at 28 d., and (2) during recovery following 28 d. stress, 20 at 7, 14 and 21 d. Adrenal response to shuttle box stress is rapid, hypertrophy becoming evident within 3 days (Fig. 1). Thereafter, the curve for stressed animals rises more slowly through remaining 25 days

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TABLE I. Comparison of Effects of 2 Stressors on Organ Weights and Total WBC of Animals Stressed for Various Periods. 10 animals/series.

Measure of stress Response and type of stressor	Period of stress 7 days Median	 Range	 14 days Median	 Range	 28 days Median	 Range
Adrenal wt/body wt						
Shuttle box stress	.344	.24-.43†	.328	.17-.43*	.405	.34-.53*
Control	.243	.16-.33	.268	.17-.40	.338	.21-.59
Confinement stress	.379	.24-.52†	.316	.18-.49	.324	.24-.44
Control	.250	.17-.30	.256	.14-.42	.242	.18-.46
Spleen wt/body wt						
Shuttle box stress	3.34	1.9-6.0	2.33	1.9-3.9†	2.63	1.5-4.6†
Control	4.14	3.2-5.1	4.14	3.3-5.2	4.84	4.0-7.3
Confinement stress	2.16	1.7-3.2	2.56	1.7-3.8	2.68	2.2-4.4
Control	2.90	1.8-3.9	2.82	2.0-4.0	3.22	1.7-5.8
Thymus wt/body wt						
Shuttle box stress	2.53	.8-3.7*	1.98	1.3-3.4†	2.49	1.4-3.5*
Control	3.17	2.5-3.7	2.99	2.8-3.8	3.08	2.4-3.6
Confinement stress	1.97	1.5-3.1†	1.82	1.5-3.1	2.08	1.0-2.9*
Control	2.96	2.1-4.4	2.52	1.5-3.5	2.96	2.0-6.7
Total WBC						
Shuttle box stress	3,650	1,500- 7,500†	3,600	3,200- 4,800†	3,050	2,000-10,600†
Control	7,100	5,100-14,200	7,100	4,600-10,500	11,400	6,500-22,700
Confinement stress	3,000	1,150- 7,300†	3,275	1,900- 5,300†	3,150	3,000-12,800*
Control	9,100	6,500-13,900	8,200	5,000- 9,700	8,800	2,750-21,900

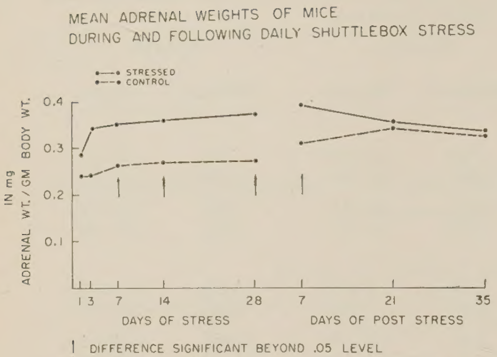
* p value of .05 to .02 by Mann-Whitney U test. † P value of .01 or greater by Mann-Whitney U test.

of stress. Differences between stressed and control adrenal values increase in significance from 7 through 28 days of stress exposure. Following stress termination, differences slowly decrease to non-significant levels. Microscopic examination indicated that stress-induced enlargement of adrenals was the result of cortical hypertrophy.

The leucocyte drop occurs even more rapidly than does adrenal hypertrophy, in some experiments after a single period of stress (Fig. 2). Difference between groups is well established at 3 days, and leucocyte values for stressed mice continue to fall throughout the 28 day stress. Recovery of leucocyte values towards control levels is rapid following stress termination. While mean differences persist, they are not significant during recovery period. The fall in total count was accounted for by decreases of polymorphonuclear leucocytes and lymphocytes, with the latter showing most pronounced changes. Increases in stress lymphocytes were observed in both stressed and control animals and were not consistently related to experimental stressors.

Spleen weights (Fig. 3) undergo initial rise following a single 6 hour period of stress. Following subsequent stress exposure progressive involution occurs which is gradual, becoming significant at 14 days and maximally significant at 28 days of stress. During recovery both stress and control values decrease significantly but differences between stress and control values are negligible. Histologically, the decrease in size of spleen was associated with marked atrophy of germinal centers of the corpuscles.

Fig. 4 shows changes in thymus weights similar to those in spleen. The initial rise following a single period of stress is again observed. Gradual involution follows continuing stress, with significant differences between experimental and control values apparent at 14 to 28 days. A tendency not statistically significant for continued decreases in both control and experimental weights occurred during recovery. Histological changes in the thymus were less pronounced than in adrenal and spleen; a tendency toward atrophy of the cortex was seen.



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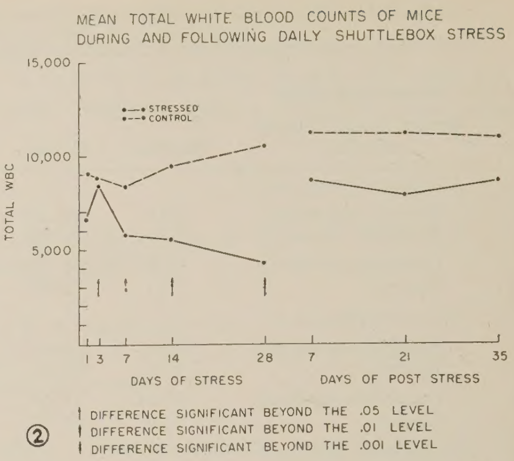

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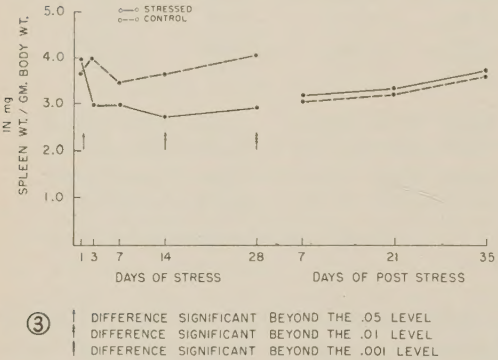

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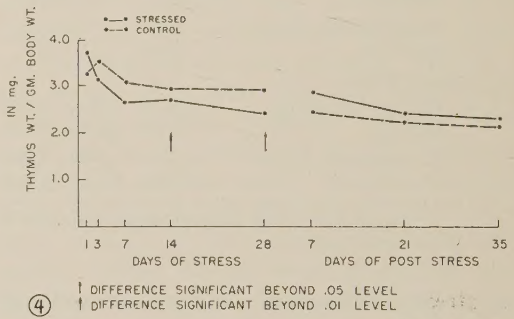

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FIG. 1. Mean adrenal weights of mice during and following daily shuttlebox stress.
FIG. 2. Mean total white blood counts of mice during and following daily shuttlebox stress.
FIG. 3. Mean spleen weights of mice during and following daily shuttlebox stress.
FIG. 4. Mean thymus weights of mice during and following daily shuttlebox stress.

Confinement stress. Organ weights and leucocyte changes were also observed in mice subjected to daily stress by confinement for 7, 14 and 28 days and data compared with shuttle box stress studies (Table I). Leucocyte and organ changes are similar in direction but smaller in magnitude and shorter in duration than changes following shuttle box stress.

Discussion. These experiments show pronounced changes in adrenal, thymus, spleen and blood leucocytes following stress of intermittent exposure either to restraint or to shuttle box training. Confinement apparently elicits changes as rapidly as shuttle box exposure, more rapidly in thymus involution; but mice tend to adapt more rapidly and more completely to restraint. This observation is supported by results of previous studies employing experimental infection(1).

Leucocyte count is most sensitive of stress response measures utilized. Changes occur consistently and more rapidly than do other measures following initial stress exposure (Fig. 2); magnitude of change is greater; and values return more rapidly following termination of stress.

That 2 dissimilar stressors elicit similar organ and leucocyte changes suggests a common mechanism. Inasmuch as confinement does not involve electrical shock, and curtails rather than enforces activity, it appears that neither electrical shock nor physical activity is crucial but that fear produced in both situations is the important factor in changes observed. Fear-motivated avoidance response is well known to resist extinction(8), and may account for the more intensive and prolonged effects elicited by shuttle box stress.

Some variation in control values is evident, (Fig. 2, 3, 4). These changes parallel, but to a lesser degree, those observed in stressed mice and may be attributed to extraneous stresses such as noises, temperature variation, individual caging and handling as has been shown by Day, *et al.*(9). The decrease in spleen and particularly thymus weights in controls during recovery is probably related to maturation.

Results differ in some respects from organ and blood changes frequently observed following stress(5). Most investigators report neutrophilic leucocytosis, and relative lymphopenia and eosinopenia. In contrast, our data show leucopenia (Fig. 2), following initial shuttle box stress, which persists through 28 days of stress and which may be related to differences in stressor application. Most studies utilized a single, short exposure to severe stressors or continuous exposure to milder stressors while our mild stressors are intermittent. Selye(5) has noted that leucopenia may follow severe stress before leucocytosis ensues and that leucopenia persists until death in animals that do not survive the alarm reaction. Our stressors, however, are neither severe nor fatal.

Perhaps relevant is the observation of Harlow and Selye(10) that counts taken immediately after exposing rats to cold show leucopenia, while leucocytosis follows in 48 hours. Our counts are ordinarily taken immediately following exposure to stress but consistent leucocytosis is not seen up to 35 days following last exposure.

Also not generally reported is initial rise in thymus and spleen weights, possibly a result of edema noted early in stress responses in these organs(11). Partial recovery of spleen and thymus weights seen during the resistance phase by others is not prominent.

Of changes observed, the rapidly occurring, persistent hypertrophy of adrenals most closely approximates stress response observed by others(5). This response appears not to account directly for increased susceptibility to viral infection, seen after 2 weeks and most marked after 4 weeks of stress(1,2). Rather, splenic atrophy is more nearly related, temporally, to susceptibility to viral infection, suggesting that impaired reticulo-endothelial activity may be an important factor. Immediate adrenal response may explain the resistance to anaphylaxis in acutely and chronically stressed animals(3).

Summary. Changes in organ weights and leucocytes following daily exposure to emotionally disturbing shuttle box or confinement stress were consistently observed. Adrenal hypertrophy and drops in circulating leucocytes were relatively rapid with significant changes observed following 3 to 7 days of stress. Involution of thymus and spleen occurred more slowly, with differences becoming maximum following 14 to 28 days of stress. Differences between experimental and control values returned to non-significant levels in 21 days following termination of stress.

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Effect of Mineralo- and Glucocorticoids on Fasted Adrenalectomized Dogs Subjected to Electroshock.* (25773)

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A basic physiological difference exists between adrenal mineralo- and glucocorticoids in that the former require an exogenous source of food and water to exhibit life-maintaining properties in adrenalectomized (adx) dogs, whereas the latter do not demand either but are capable of restoring normal activity and vigor to the starved, dehydrated animal despite continued withholding of fluid and nutrients during recovery period(1-3). The following experiments were designed to explore further this significant difference between the 2 steroid types with special reference to the role of glucocorticoids in restoring to normal health, fasted adx dogs exhibiting severe symptoms of insufficiency as a result of stress induced by electroshock.

Methods. Electroshock was employed as a convenient stressor to induce circulatory failure in adx dogs. Animals adequately maintained by daily i.m. injections of 0.5 mg DCA were subjected to severe electroshock; steroid was withheld 2-3 days and the dog fasted 24 hours before applying electric current. Shock was produced by passing 150-180 milliamperes of electric current through the brain for 0.2 second and the procedure repeated within 30 minutes in most cases. The animals exhibited convulsions, muscle rigidity, salivation and loss of consciousness. These manifestations were accompanied by micturition and defecation. Gross symptoms disappeared within 5-10 minutes. Blood for sampling (37 cc) was withdrawn 15-30 minutes later, the urinary bladder drained, rinsed with distilled water and the dogs placed in metabolism cages without access to food or water for an additional 48-72 hours. Non-adx animals are relatively insensitive to rapidly repeated, severe electroshock; the initial reaction although violent, is evanescent, recovery is complete and there are no after effects. Response of the fasted adx dogs maintained on DCA to application of electrical current to the brain is quite different. They rapidly recover from the convulsive seizure then slowly develop a shock-like syndrome during the ensuing 24-48 hours which terminates fatally in untreated animals.

Results. I. Failure of mineralocorticoids (DOC), to revive fasted adrenalectomized, electroshocked dog from circulatory failure. Pertinent data are presented in Tables IA and IIA. During the 15-30 minute interval after shock mean arterial pressure declined. Sharp rises usually occurred in blood sugar and blood urea nitrogen of DOC-treated animals. Hemoconcentration was present and acidosis evident, as indicated by fall in blood pH and CO_2 content. There was reduction in plasma volume and an increase of plasma Na which in Dog 1, later attained an extreme value. During the following 24 hours these deviations from control values occasionally became more marked. At termination of the 24th hour after administering electroshock, blood was withdrawn for sampling; treatment with solubilized DOC was then started and 30-50 mg were given i.v. in divided doses; Dog 3 was exceptional in that mineralocorticoid was given 5 hours after shock. The steroid failed to alleviate symptoms or improve the clinical condition. Plasma K continued to rise in Dog 1 whereas renal elimination of this ion practically ceased Severe symptoms of hyperkalemia (*e.g.*, loss of control of the hind legs) were present. This animal's condition was so serious the experiment was terminated. He was restored to normal activity within 6 hours by infusion of 250 cc of 1% NaCl plus 2% of Na HCO_3 and 10 mg of 2-methyl-9α-fluorohydrocortisone. This therapy provoked K

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† New Jersey Heart Assn.

TABLE I. Effect of Severe Electroshock on Active, Vigorous, Adrenalectomized Dogs Maintained on Desoxycorticosterone.

Time (hr), condition of animal		Body wt, kg	BP, mm Hg	Blood urea N, mg %	Hb, g %	Hmct, %	RBC 10^6, mm^3	Blood sugar, mg %	pH	Plasma CO_2 vol, %	Plasma cc/kg	Vol, % change	Steroid, mg 24 hr
A. Failure of mineralocorticoid (DOC) to prevent circulatory failure in electroshocked adrenalectomized dogs deprived of food and water. Dogs 1-3.													
Initial	N	19.3	116	23.4	12.4	38.5	6.11	79	7.32	44.3	53.9		
15 min.	S	19.1	63	38.6	16.2	46.6	7.92	90	7.15	34.9	30.0	−44.3	
24 hr	S	18.9	79	67.6	16.0	44.7	7.29	71	7.21	32.5	29.7	−44.8	30*
48	S	18.4	42	87.5	16.7	46.1	7.21	70	7.23	34.6	30.2	−43.9	
Initial	N	26.7	130	24.0	13.7	38.8	5.62	79			43.9		
30 min.	S	26.5	95	24.1	15.6	45.4	6.34	104			36.6	−16.6	
24 hr	S	25.4	92	30.2	15.6	45.6	6.32	80			25.9	−41.0	50*
48	S	24.8	80	48.0	15.4	43.8	6.14	92			28.6	−34.8	100‡
72	R	24.2	96	28.2	13.8	39.7	5.79	126			44.3	+71.0	
Initial	N	23.1	122	21.1	15.4	40.0	5.73	76			46.3		
5 hr	S	22.0	90	29.1	14.7	39.5	5.84	95			34.1	−26.3	50†
24	S	21.0	70	40.4	13.7	38.1	5.76	91			34.0	−26.5	100‡
48	R	21.3	90	21.9	11.7	34.6	4.32	108			45.5	+33.8	
B. Restoration of electroshocked adrenalectomized dogs to normal with glucocorticoids despite lack of food and water. Dogs. 4-6.													
Initial	N	21.5	110	19.7	11.5	32.9	4.99	86	7.38	51.8	49.3		
15 min.	S	21.3	95	28.3	12.9	36.3	5.60	89	7.00	41.0	42.8	−13.1	
24 hr	S	20.6	87	29.0	12.8	33.1	5.13	80	7.31	47.9	37.6	−23.7	13§
48	R	19.7	106	22.3	9.9	26.4	4.42	95	7.41	46.8	57.2	+52.7	
Initial	N	20.2	112	23.1	10.4	30.1	4.50	86	7.33	44.7	48.4		
15 min.	S	20.0	98	21.4	12.8	36.3	6.08	96	7.20	34.8	42.5	−12.7	
24 hr	S	19.3	78	26.5	12.9	37.0	5.61	77	7.36	44.6	34.7	−28.3	50‖
48	R	18.2	110	24.1	10.1	29.9	4.75	100	7.32	40.2	43.0	+23.1	
Initial	N	27.1	102	20.2	11.7	31.1	4.53	84	7.32	47.7	44.7		
15 min.	S	26.6	100	22.3	12.8	35.4	5.80	96	7.14	25.8	33.8	−24.3	
24 hr	S	25.5	80	25.5	13.6	36.1	5.18	75	7.32	46.4	32.1	−28.1	50‖
48	R	24.7	88	18.8	11.1	29.8	4.12	98	7.37	42.6	46.6	+45.1	

* DOC i.v. in 2 divided doses 24 hr after electroshock.

† *Idem* 5 hr after electroshock.

‡ 1-dehydrohydrocortisone hemisuccinate i.v. in 2 doses 24 or 48 hr after electroshock.

§ 2-methyl-9 α-fluorohydrocortisone i.v. in 2 doses 24 hr after electroshock.

‖ 50 mg 1-dehydrohydrocortisone hemisuccinate i.v. in 2 doses 24 hr after electroshock.

− = Decrease in plasma vol following electroshock and DOC therapy. + = Increase in plasma vol after glucocorticoid injections. N = Normal. S = Shock. R = Recovery.

diuresis which reduced plasma K from 8.93 to 4.6 meq/l. Dogs 2 and 3 recovered rapidly following injection of glucocorticoid despite continued deprivation of food and water. Large amounts of K were excreted accompanied by reduction in plasma K. Table II.

II. Restoration of fasted electroshocked adx dog to normal health with glucocorticoids. Alterations in blood pressure, blood constituents and plasma volume produced by electroshock at 15 minutes and 24 hours differed only in degree from those discussed for the DOC series (Tables IB and IIB). The changes were milder since near loss of one of the DOC-treated dogs showed the necessity for initiating restorative measures before serious symptoms developed. Glucocorticoids were given i.v. in divided doses beginning 24 hours after electroshock and continued until recovery which was complete 24 hours later. Unlike the DOC-treated dogs, these animals showed a prompt return to control or near control values of arterial pressure; hemoconcentration was much reduced and striking increases in plasma volume occurred. Plasma Na, Cl and K remained at or near levels observed before therapy but in view of the expansion of plasma volume the quantity of these ions must have increased. Fluid and electrolyte diuresis appeared with renal excretion of enhanced quantities of Na, Cl and K in those dogs receiving 1-dehydrohydrocortisone but retention of Na and Cl and elimination of K occurred in the one animal injected

TABLE II. Plasma and Urine Electrolytes of Fasted Electroshocked Adrenalectomized Dogs.

Time (hr), condition of animal		Plasma electrolytes, meq/l			Urine vol, cc	Urine electrolytes, meq		
		Na	Cl	K		Na	Cl	K
A. Treated with mineralocorticoid. Dogs 1-3.								
Initial	N	144.0	117.9	4.40				
15 min.	S	146.0	110.8	4.62				
24 hr	S	150.0	114.9	7.72	120	6.7	11.3	2.53*
48	S	162.0	118.2	8.93	24	2.4	4.2	1.34
				Total	144	9.1	15.5	3.87
Initial	N	141.0	111.0	5.03				
30 min.	S	142.0	109.4	5.03				
24 hr	S	143.0	111.5	5.46	340	40.12	32.6	6.08*
48	S	147.0	115.9	5.20	250	3.28	2.78	14.83‡
72	R	141.0	119.2	4.38	250	1.42	.56	29.86
				Total	840	44.82	35.9	50.77
Initial	N	141.0	111.1	4.64				
5 hr	S	141.0	111.1	4.66				†
24	S	141.5	112.8	5.01	89	5.3	4.0	9.6 ‡
48	R	142.0	115.5	3.73	270	2.3	4.2	41.7
				Total	359	7.6	8.2	51.3
B. Treated with glucocorticoids. Dogs 4-6.								
Initial	N	147.5	119.0	4.06				
15 min.	S	150.0	117.3	4.22				
24 hr	S	150.5	119.7	4.95	220	44.2	34.7	7.0 §
48	R	152.0	116.9	4.38	425	5.3	16.4	48.6
				Total	645	49.5	51.1	55.6
Initial	N	148.0	116.8	4.06				
15 min.	S	150.5	117.9	4.47				
24 hr	S	151.0	117.6	4.93	222	28.4	26.2	14.1 ‖
48	R	149.0	122.1	4.95	370	47.0	26.3	37.4
				Total	592	75.4	52.5	51.5
Initial	N	144.0	116.0	4.48				
15 min.	S	147.0	115.0	4.62				
24 hr	S	147.5	115.9	4.97	205	43.1	40.2	12.6 ‖
48	R	147.5	112.8	4.60	675	93.9	71.0	54.4
				Total	880	137.0	111.2	67.0

* 30-50 mg DOC in 2 divided doses 24 hr after electroshock.

† 50 mg DOC in 2 divided doses 5 hr after electroshock.

‡ 100 mg 1-dehydrohydrocortisone hemisuccinate i.v. in 2 divided doses 48 hr after shock.

§ 13 mg 2-methyl-9α-fluorohydrocortisone 24 hr after shock.

‖ 50 mg 1-dehydrohydrocortisone hemisuccinate i.v. in 2 divided doses 24 hr after shock.

N = Normal. S = Shock. R = Recovery.

with 2-methyl-9α-fluorohydrocortisone — a steroid which possesses both glucocorticoid and mineralocorticoid activity in marked degree (Table IIB, Dog 4). Urinary loss of Na and K during the 24 hour period of recovery after receiving glucocorticoids was for Dogs 4-6: 5.3, 47.0 and 93.9 meq of Na and 48.6, 37.4 and 54.4 meq of K. Since food and water were unavailable and plasma electrolytes remained elevated whereas the plasma volume had expanded, it seems justifiable to conclude that some of these ions, especially K, were derived from cells accompanied by large volumes of water shifted into the intravascular space. The source of the considerable quantities of Na and Cl poured out in the urine is not so evident, although some apparently were also derived from cells.

Discussion. An immediate response of mammalian species to electroshock is marked disturbance of the electrolyte pattern of the brain cells. K shifts out of the cells and is replaced by Na during the convulsions. When this phase has ceased, a reverse shift of ions and water is initiated and Na and K resume their usual extra- and intracellular positions (4,5). Acidosis, increased plasma Na, decline in plasma volume and rapid redistribution of

TABLE III. Water and Electrolytes Mobilized from Extravascular Sources Following Glucocorticoid Therapy. (Amounts in expanded vascular compartment and urine combined.)

Dog. No.	Water, cc	Na^+, meq	K^+, meq
4	777	60	50
5	483	63	38
6	1007	143	55

body water characterized by contraction of the extracellular space have been reported to occur after electroshock(6-8). All investigators cited comment that the changes enumerated are transitory and rapidly disappear upon cessation of convulsions. However, data in Tables I and II demonstrate that fall in arterial pressure, plasma volume and increased hemoconcentration, induced by electroshock in fasted adx dogs following withdrawal of DCA for several days, are not merely temporary but are changes the untreated animal is unable to reverse spontaneously. Solubilized mineralocorticoids such as DOC and aldosterone when administered i.v. in relatively enormous doses are ineffective for reviving fasted adx dogs from shock or insufficiency whereas glucocorticoids are extremely effective and readily restore activity and vigor to the starved, dehydrated animal prostrate from insufficiency.

Data presented in Tables I and II demonstrate the effectiveness of glucocorticoids in contrast to desoxycorticosterone for reviving these seriously ill animals from the stress of electroshock. Following injection of this type steroid, arterial pressure and plasma volume increased markedly, hemoconcentration was sharply reduced but alterations of plasma K were not a consistent finding. Although the concentrations of plasma Na and Cl did not show significant changes the total quantity of these ions in the intravascular compartment increased considerably (Table III). Since exogenous sources of water and electrolyte were lacking during recovery obviously large amounts of these substances must have been mobilized from extravascular sources and shifted into the *intravascular* compartment. Approximate values for the quantities of water, Na and K mobilized by the glucocorticoid (Dogs 4-6, Tables I and II) are shown in Table III.

The dramatic and rapid recovery of the animals is due, apparently, to 1) activity of glucocorticoids in mobilizing fluid and certain electrolytes from extravascular sites and redistributing them to the intravascular compartment and 2) stimulation of renal mechanism to excrete excess water, Na and K mobilized as a result of administering the steroid. Thus adrenal C_{11} oxysteroids, in contrast to desoxycorticosterone and other mineralocorticoids, appear to be a vital part of the mechanism which maintains the internal water and electrolyte balance of the vertebrate organism. However, the exact role they play in this important function remains unknown.

Summary. Electroshock induces circulatory failure in fasted adrenalectomized dogs maintained on mineralocorticoid. Treatment with desoxycorticosterone is ineffective and animals succumb exhibiting retention of Na, Cl and renal elimination of but small amounts of K. Despite continued fasting, glucocorticoid injections bring about rapid return of normal activity and vigor accompanied by increased arterial pressure, plasma volume, and decreases in hemoconcentration. Plasma Na and Cl concentrations showed little or no changes but the total quantity of these ions in the intravascular compartment increased significantly. The data indicate that C_{11} oxysteroids mobilize large amounts of water and electrolyte from extravascular sources of the adrenalectomized dog prostrate from insufficiency and shift them into the intravascular compartment thereby bringing about dramatic and rapid recovery. Thus glucocorticoids, in contrast to mineralocorticoids, are apparently a vital part of the mechanism which maintains the internal water and electrolyte balance of vertebrate organisms although the precise role they play in this important function is unknown.

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Effect of Reserpine on Adrenalectomized Rats.* (25774)

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Gaunt *et al.*(1) noted that doses of reserpine ranging upwards from 50 μg/kg/day reduced survival time of adrenalectomized rats. This might be due to an increased requirement for cortical hormones or to release of serotonin from tissues(2). Jaques, Mogenson and Fisher(3) showed that hemorrhage occurred readily when adrenalectomized rats were given dicumarol, all dying of hemorrhage while controls all survived. This was explained by the observation(4,5) that interference with any 2 of the 3 major hemostatic lines of defense (blood coagulation, platelet plugging, vascular integrity and response) present an overwhelming hemorrhagic defect. The work of Correll *et al.*(6) and Zucker and Borrelli(7) indicated that platelet serotonin is involved in normal hemostatic mechanisms, and Jaques and Fisher(4) showed that reserpine produced hemorrhage in rabbits subjected to stress. The experiments reported were undertaken to compare hemorrhagic death in adrenalectomized rats treated with reserpine, with that already studied in other experiments using these treatments.

Methods. Adrenalectomized rats were maintained on 0.9% saline and received hydrocortisone (1 mg/100 g/day) for 3 days post-operatively. Treatments were started one week after operation, as follows—reserpine 2 mg/kg intraperitoneally, desoxycorticosterone acetate (DOCA) 2.5 mg/kg daily subcutaneously. The animals were examined daily. Mortality was recorded as number of deaths to the tenth day. A careful autopsy was made.

Results. Groups of approximately 25 rats (adrenalectomized and sham-operated) were divided into subgroups receiving reserpine and no reserpine. The experiment was then repeated with DOCA given at various time intervals. Number of animals, respective treatments, and mortality are shown in Table I.

Adrenalectomy + Reserpine. There was a profound diarrhea and intense gastrointestinal discomfort in animals of Group 1. Half of them died within 6 hours, others died within 24 hours to give a mortality of 80% (16 rats). In every case diarrhea was present. Feces were eliminated and thin mucus kept issuing from the anus. The presence of gross blood in feces and/or intestinal lumen was observed in 25% of the post mortem examinations.

TABLE I. Mortality of Adrenalectomized Rats Treated with Reserpine.

	No.	Died	% mortality
Adrenalectomized + reserpine	20	16	80
Adrenalectomized only	18	3	17
Sham operated + reserpine	25	1	4
Sham operated only	21	1	5
Adrenalectomized DOCA for 8 days, reserpine on 6th day	10	1	10
Adrenalectomized DOCA for 4 days, reserpine on 6th day	10	4	40
Adrenalectomized DOCA for 4 days, control	10	1	10
Adrenalectomized DOCA for 8 days, control	9	2	22
Sham operated DOCA for 8 days, control	7	0	0
Sham operated DOCA for 8 days, reserpine on 6th day	10	1	10

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* Supported by Natl. Research Council of Canada.

The lungs were clear indicating that death following reserpine was not due to pulmonary complications. Heart and liver appeared normal but with some engorgement. In almost every animal, the testes were found withdrawn into the abdominal cavity, giving a picture of distress. All animals receiving this dose of reserpine were tranquilized but adrenalectomized animals alone showed the extreme intestinal symptoms. The sham-operated animals receiving reserpine (Group 3) showed far less severe diarrhea than Group 1 and there was no obvious blood in the stools. Only one animal died. It showed a badly hemorrhaged intestine with melena. The lungs, heart, and liver were normal. In control animals (Groups 2 and 4), there was no diarrhea. Two adrenalectomized animals died between 24-48 hours. One sham-operated animal died on the ninth day of apparently self inflicted wounds and/or bleeding from the tail and forefoot. The post mortem appearance was quite different from the reserpine-treated animals. Nasal bleeding and lung hemorrhage were observed suggesting respiratory involvement. The third adrenalectomized animal which died showed nothing unusual.

Adrenalectomy + DOCA + Reserpine. When adrenalectomized rats were maintained on DOCA throughout, (Group 5) there was only one death, indicating that DOCA afforded considerable protection to such animals receiving reserpine. Withdrawal of DOCA 2 days before reserpine (Group 6) resulted in greater mortality. Gross pathology was similar to that (Group 1) of adrenalectomized animals dying after reserpine. Two deaths occurred within 6 hours and 2 occurred at 16 and 40 hours. Diarrhea was evident and on testing the feces or intestinal contents for occult fecal blood three strong positives were obtained. None showed grossly hemorrhagic lungs although one or 2 petechiae were noticed, which could not have been the cause of death. A few deaths were observed in the control groups (Groups 7, 8, 9). Gastrointestinal symptoms and stools positive for blood were not observed in these animals. In those that died there was pronounced lung congestion and hemorrhage so that pulmonary complications were evidently the cause of death. Sham-operated rats (Group 10) receiving reserpine and DOCA showed nothing unusual. One animal died 16 hours after reserpine and showed an intense loss of blood via intestine, lung hemorrhages and withdrawn testes. The other 9 animals exhibited no signs of gastrointestinal distress.

Discussion. Diarrhea and intestinal hemorrhage appear responsible for death following this dose of reserpine in adrenalectomized rats. Plummer *et al.*(8) describes activity of reserpine on the gastrointestinal tract as augmenting both secretory and motor activity, presumably due to release of serotonin from gastrointestinal mucosa. Van Cauwenberge and Jaques(5) showed that when dicumarol was given to adrenalectomized rats there was considerable mortality (40%). Hemorrhage was not prominent but adrenalectomized rats maintained on DOCA, when given dicumarol, all died of spontaneous hemorrhage; maintained on cortisone all survived anticoagulant treatment. Normal rats receiving both reserpine and dicumarol showed a high mortality (50%) from hemorrhage. In all these experiments, spontaneous hemorrhage was quite widespread through the body and was produced by a double defect in hemostasis—coagulation affected by dicumarol, capillary resistance by adrenalectomy(9), platelets by reserpine. Cortisone and DOCA respectively corrected and increased the vascular defect in adrenalectomized animals.

Adrenalectomy and reserpine caused 80% mortality. The post mortem revealed much loss of fluid and blood *via* intestine. Hemorrhage was always in close association with intestinal tract, and was not found elsewhere probably due to the short survival time. Fluid loss was reduced following administration of DOCA. The difference in observations between reserpine-dicumarol-normal rat and reserpine-adrenalectomized rat suggests that the intestine is more sensitive to reserpine (serotonin) in the adrenalectomized rat. Selye and Heuser(10) mention that an inadequate adrenal defense mechanism appears to predispose the tissues of the gastrointestinal tract to ulceration. The known defects in water and electrolyte balance in the adrenalectomized rat and correction of these by DOCA could also explain the present results with sudden loss of mucosal serotonin causing intraintestinal loss of water. Cause of death in adrenalectomized animals receiving reserpine therefore is not as simple as that of normal rats receiving dicumarol and reserpine, or adrenalectomized rats receiving dicumarol, where there is a clear interference with 2 mechanisms of hemostasis simultaneously. However, it seems reasonable to accept that part of the situations which are common—increased capillary fragility and serotonin displacement, contribute to the hemorrhage. The marked gastrointestinal activity of reserpine in the adrenalectomized animal will mean this must be main site of hemorrhage, and the fact that platelet physiology has been disturbed may possibly mean that when intraintestinal hemorrhage occurs, the platelet plugging required for hemostasis cannot follow.

Summary. Reserpine given to adrenalectomized rats produced diarrhea and gastrointestinal hemorrhage with high mortality. This was not observed in sham-operated animals. Maintenance on desoxycorticosterone protected the animals from this action of reserpine.

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Cytostatic Activities of Steroidal Estrogens against Zebra-Fish Embryos. (25775)

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To find a superior estrogen for chemotherapy, we have studied a large list of steroidal estrogens for their cytostatic effects on the egg of the zebra-fish (*Brachydanio rerio, Hamilton*). Of the 14 steroidal estrogens studied, we have also tested the 3-methyl and 3-ethyl ethers of each. Some of these ethers are reported here for the first time.

Materials and methods. The method was a modification of one previously described(1). Our present practice is to use 10 embryos (8-64 cell stages) in 50 ml of culture solution exposed at 26°C for 24 hrs. Each test culture is replicated and at least 3 replicated tests are used to determine minimum concentration (in parts per million) of the steroid which affects 50% of the embryos (ED/50/24). Thus, a minimum of 50-60 embryos was used in testing each concentration.

Results. The results are summarized in Table I.

Discussion. As measured in the restraint of cell division in the freshly-fertilized zebra-fish egg, 17-ketosteroids are in general less cytostatic than 17-hydroxysteroids. The one exception is equilenin, which produced a curious type of cytostasis. The equine estrogens

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TABLE I. Comparative Cytostatic Activities of Steroidal Estrogens on the Zebra-Fish Embryo.

		ED/50/24		
	Estrogen	Free phenol, ppm.	3-Methyl ether, ppm.	3-Ethyl ether, ppm.
Bi-functional	Estrone-17 (U.S.P.)	Inactive	2–4	1–2
	Estradiol-17β (U.S.P.)	2–3	1–2	.5–1
	” -17α	2	2–3 (D)	.5–1 (D)
	Estrone-16	Inactive	2–4	Inactive
	Estradiol-16β	2	.5–1	”
	” -16α	1	1–2	”
	Equilin	Inactive	1–3 (D)	” (>20)
	17-Dihydroequilin-17β	1–2 (D)	1–2	.5
	Equilenin	.5–1 (D)	Inactive	Inactive
	17-Dihydroequilenin-17β	.5–1 (D)	.5–1 (D)	1–2 (D)
Tri-functional	16-Keto-estrone	Inactive	Inactive	2–3
	16-Keto-estradiol-17β	”	”	Inactive
	16-Epiestriol	”	”	”
	Estriol	”	”	”

ED/50/24 means concentration in which 50% of embryos show effects of drug after 24 hr of exposure. Unless indicated otherwise, effects are considered to be cytostatic. This is usually indicated by death of embryo or abnormal development. In cases indicated by (D) effect is retardation of development. *Inactive* means that ED/50/24 was clearly greater than 2 ppm. Often partial insolubility of steroid results at concentrations greater than 2 ppm.

often caused only retardation of development with no lethality.

Bi-functional estrogens are considerably more active than tri-functional estrogens. This is in curious contrast to the situation which obtains in measurement of the anti-mitotic activity against the chick-embryo fibroblast in tissue culture. We have reported(2) the extremely powerful effects of certain tri-functional estrogens. Estriol, however, had very weak anti-mitotic effects against the fibroblast.

16-Oxygenated estrogens were found to be more cytostatic than 17-oxygenated estrogens when the steroidal C_3 position is hydroxyl or methoxyl; this situation, however, is reversed in the case of 3-ethoxyl substitution.

In agreement with the findings of Druckrey, Danneberg and Schmähl(3), who used the sea urchin egg, we have found no relationship whatever between estrogenic hormone potency and cytostatic potency. As an illustration, the 3-methyl ether of estradiol-16β is 3 to 4 times more cytostatic than natural estradiol, but the latter is at least one thousand times the more potent by the Allen-Doisy assay.

Summary. The cytostatic effects of 14 steroidal estrogens and their 3-methyl and 3-ethyl ethers have been studied using the embryo of zebra-fish (*Brachydanio rerio*) as test object. Of estrogens tested, 3-ethyl ether of 17-dihydroequilin-17β was the most active.

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Induction of Mammary Growth and Lactation in Rabbits with Epinephrine, Acetylcholine and Serotonin.* (25776)

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Recently we reported that numerous drugs, neurohormones, nonspecific stresses and other factors could induce mammary growth and lactation in estrogen-primed rats, and that some of these could maintain mammary secretion in postpartum rats after litter removal (1-7). These agents are believed to evoke release of prolactin and probably ACTH from the anterior pituitary *via* autonomic and central nervous system. Epinephrine, acetylcholine and serotonin were among the most effective agents used and are of particular interest since they are natural substances in the body, and may normally mediate some effects of ovarian hormones or of other physiological stimuli in producing mammary growth and lactation. It was of interest therefore to see whether these 3 agents could induce milk secretion and mammary growth in the rabbit, since in pseudopregnant rabbits such responses are readily elicited by prolactin injections(8).

Methods. A total of 38 female, predominantly New Zealand White rabbits, ranging from 3.1-4.4 kilos, were used. All rabbits were first injected subcutaneously with 0.2 mg estradiol in corn oil daily for 15 days. For the next 5 days they were each injected subcutaneously as follows: controls (11 rabbits), 0.85% saline once daily; epinephrine in oil[‡] (8 rabbits), 0.25 mg/kg BW twice daily; acetylcholine iodide[§] (8 rabbits), 12.5 mg/kg BW twice daily; serotonin creatinine sulfate[‖] (11 rabbits), 5 mg serotonin base/kg BW once daily. On day after last injection (21st day of experiment), attempts were made to express milk manually from nipples of each rabbit, and results recorded. Rabbits were then killed and mammary glands exposed for gross observation. Ratings of 0-5 were assigned each rabbit for mammary growth and lactation, depending on degree of mammary development and amount of milk present. Mammary tissue was also removed and prepared by standard histological technics for microscopic examination.

Results. Milk could be expressed from nipples of only 1 of 11 saline-injected controls, and this animal also showed slight lobulo-alveolar development. An insignificant volume of thin watery secretion could be expressed from nipples of 3 of the other 10 controls, and only extended ducts and end buds could be seen in the mammary glands. Milk was expressed from nipples of 6 out of 8 epinephrine-treated rabbits, and they were assigned an average lactation rating of 2. Good lobulo-alveolar mammary development was present in these rabbits (avg rating = 2.7). Microscopically, the alveoli were distended with secretion. Acetylcholine-injected rabbits showed a lesser response, with gross lactation in 5 out of 8 (avg rating = 1.5) and only fair lobulo-alveolar growth (av. rating = 2). The best responses were seen in serotonin-injected rabbits, 9 out of 11 showing gross lactation (avg. rating = 2.8) and good to excellent lobulo-alveolar growth (avg. rating = 3.1). In general, microscopic examination of stained sections of mammary tissue from these rabbits confirmed gross observations. Mammary responses to different treatments are shown in Fig. 1-4.

Intense salivation was usually observed in acetylcholine-injected rabbits a few minutes after injection of drug. In preliminary trials with higher doses of acetylcholine, convulsions were frequently seen and often were followed by comatose state or death. No obvious symptoms were noted following epinephrine or serotonin administration.

Discussion. The present results in rabbits demonstrate that epinephrine, acetylcholine

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[‡] Parke, Davis and Co.

[§] Eastman Organic Chemicals

[‖] Nutritional Biochemicals Corp.

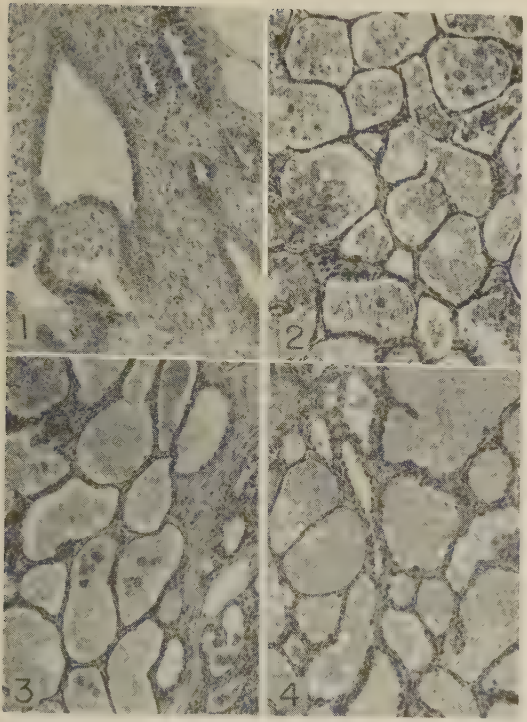

FIG. 1-4. Histological sections from mammary glands of estrogen-primed rabbits treated for 5 days with (1) saline, (2) epinephrine in oil, (3) acetylcholine iodide, (4) serotonin creatine sulfate. × 70.

and serotonin can produce lactation and mammary growth in estrogen-primed rabbits, as previously reported in estrogen-primed rats. Gross lactation was observed in ducts and lobules of these rabbits and milk could readily be expressed from the nipples, whereas only histological evidence of mammary secretion was seen in rats. Although amounts of milk noted in mammary glands of these rabbits were not equal to that usually seen after parturition, it is probable that better initial development of mammary glands, and perhaps longer treatment with drugs, would have produced greater milk production. The 3 agents produced mammary lobulo-alveolar growth as well as lactation, since only ducts and end buds were present in the saline-injected controls.

The mechanisms by which the 3 drugs induced prolactin release from the anterior pituitary remain to be determined. That anterior pituitary is essential is shown by negative mammary responses to these 3 drugs when injected into rats hypophysectomized after estrogen-priming (unpublished observations). They may act directly on the anterior pituitary, on hypothalamus or on other parts of CNS. Rat hypothalamic tissue has recently been shown to induce mammary secretion in intact, estrogen-primed rats(5), which suggests but does not necessarily prove that the hypothalamus contains a prolactin-releasing factor. On the other hand, there is considerable evidence that the hypothalamus normally inhibits prolactin release(9), and the 3 agents may therefore depress the hypothalamic center or centers concerned with prolactin release. Participation of the autonomic nervous system in release of prolactin is suggested by reports that bilateral cervical sympathectomy in the goat leads to marked and prolonged inhibition of milk secretion(10), and interruption of sympathetic pathways prevents induction of pseudopregnancy in rats by electrical stimulation of the uterine cervix(11).

Summary. 1. Thirty-eight mature female, predominantly New Zealand White rabbits, were injected subcutaneously daily with 0.2 mg estradiol for 15 days to develop the mammary duct system. They were then divided into 4 groups and injected subcutaneously as follows for 5 days: 0.85% saline; 0.25 mg epinephrine in oil twice daily; 12.5 mg acetylcholine iodide/kg BW twice daily; 5 mg serotonin base/kg BW once daily. On 21st day the rabbits were killed and mammary glands examined for growth and lactation. 2. Only 1 of 11 saline-injected rabbits showed gross lactation and lobulo-alveolar development, in contrast to 6 of 8 epinephrine-treated rabbits, 5 of 8 acetylcholine-injected rabbits and 9 of 11 rabbits treated with serotonin. The latter drug induced the greatest, and acetylcholine the least degree of mammary growth and lactation. 3. These results demonstrate that the 3 drugs in pharmacological doses can induce prolactin release from the anterior pituitary of rabbits.

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Secretion of Dietary Strontium 90 and Calcium in Human Milk.*† (25777)

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The preferential utilization of calcium over strontium by man and many species of animals has been well studied; the concepts and magnitude of the differential behavior have been generally established(1-7). For the cow and goat under usual management, it has been shown that Sr/Ca ratio in the milk is about 0.1 of that in the diet, with values from various parts of the world falling well within the range 0.09 to 0.16(4,6,7). No direct evidence, to our knowledge, has been reported for strontium-calcium relationships between diet and human milk. It seemed desirable to obtain data on this point (a) to see if the same general relationships hold for man as for animals, since much information is being acquired under carefully controlled conditions in animals and any basis for judicious extrapolation to man might be helpful; (b) to permit estimations or predictions of Sr^{90}/Ca ratios or Sr^{90} levels in human milk from knowledge or predictions of dietary levels; and (c) to permit estimations of relationships between Sr/Ca of diet and Sr/Ca of the body in healthy individuals; this type of information has been difficult to obtain. It became possible to measure these relationships in normal healthy women by making use of Sr^{90} levels that existed in our diet during May 1959. In principle, it appears that calcium and strontium of milk are derived under normal conditions largely from calcium and strontium of the contemporary diet with a small contribution from skeletal reserves, and this most likely from regions that have been labeled recently (4,8). Thus, a study was done in which a known diet was fed to 5 women starting immediately postpartum; after a conditioning period of about 20 days, a 5-day collection of food equivalent to that eaten and a 5-day collection of all milk were obtained for analysis.

Methods. Each of the 5 subjects began 30-day participation immediately postpartum. The program was as indicated in Table I. If necessary, lactation was enhanced by pumping the breasts to assure a good volume of milk secretion and an active lactation for at least 30 days. Breast milk secreted during the last 5 days of the 30-day period was collected for analysis. a) *Dietary control.* The hospital diet was consumed during the first 5 or 6 days postpartum. On leaving the hospital the subjects went to an apartment where they were

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* Actual data in this experiment refer essentially to (Sr^{90}/Ca of extracellular fluids)/(Sr^{90}/Ca of diet) during period of observation. The same $OR_{body/diet}$, however, would be expected to hold over most, if not all, of the life of the individual, as shown by previous animal experiments where short-term double tracer studies have been compared with lifetime feeding(1,2).

† We acknowledge cooperation of Clement A. Smith, Harvard Med. School, without whose intermediation, arrangements for the study would have been impossible, and of Charles L. Easterday, who supplied medical supervision of subjects and counseled regarding composition and control of diet; also of Ira B. Whitney and Edward P. Hardy, Health and Safety Lab., who tabulated much of the data.

TABLE I. Description of Subjects and Protocol.

Subject No.	I	II	III	IV	V
Age (yr)	20	17	22	18	22
Ht (in)	66	66	67	64.8	67.5
Wt (lb)	115	131	140	125	137
Physical condition at delivery	Good	Good	Good	Good	Good
Health during exp.	"	"	"	"	"
History of previous lactation	Grav. I Para. I Bottle fed by choice	Grav. I Para. I Nursed by choice	Grav. II Para. I Bottle fed by choice	Grav. I Para. I Bottle fed by choice	Grav. II Para. II Bottle fed by choice
Dates (all 1959)					
Under hospital care	May 10–15	Apr. 26–May 4	Apr. 11–20	May 8–15	Apr. 23–28
Start on controlled diet	May 15	May 4	Apr. 20	May 15	Apr. 28
Food consumption measured	May 25–June 8	May 10–26	Apr. 27–May 11	May 24–June 7	May 7–22
Food equivalent to that eaten saved for analysis, and total breast milk collected	June 4–8	May 22–26	May 7–11	June 3–7	May 18–22

served meals prepared especially for them by a trained technical group. This diet conformed to accepted standards for use during the lactation period and was approved by the physician responsible for their medical supervision. The objective was to feed a diet sufficiently constant to establish steady state with respect to calcium and strontium 90. Steps were taken to avoid sporadic variations in strontium 90 intake. Since vegetables may be rather variable in strontium 90 content, a supply of frozen and canned foods was provided in advance. This permitted all subjects to be fed from one lot throughout the period. Meat and milk were procured from day to day. The diet was not unique except that bread was excluded because preliminary data indicated possible wide fluctuations in strontium 90 concentration. It was not possible to dictate amount of food consumed each day but, although amount eaten was determined voluntarily, it was hoped that these healthy young women would maintain a rather uniform intake. From the 16th to the 30th day postpartum, each kind of food in a serving was weighed before and after the meal to determine weight consumed. Although there was considerable variation from day to day in both periods, average daily intake of the various foods did not differ markedly in the 10-day preliminary period and the 5-day collection period. This was notably the case with respect to milk products and vegetables, the 2 principal dietary sources of strontium 90. It is believed, therefore, that the subjects were reasonably in steady state in regard to diet, circulating body fluids, and accessible parts of the skeleton after the 10-day preliminary period and that conditions remained substantially the same during the 5-day collection period. b) *Collection of ingested diet and breast milk.* During the last 5 days, the collection period, a weight of each dietary item equal to amount consumed was placed in a polyethylene bottle and stored at about –15°C. All of the breast milk secreted during collection period was composited in another polyethylene bottle and stored in frozen condition. c) *Chemical analysis.* Collected food and breast milk were evaporated to dryness and ashed in a muffle at about 600°C until a white ash was obtained. The food ash was fused with sodium carbonate and the mixture dissolved in hydrochloric acid. The milk ash was dissolved directly in hydrochloric acid. The 2 quantitative estimations of calcium and strontium 90 were made by methods described by Harley and Whitney(9). d) *Expression of results.* Sufficient data are given to permit calculations on various bases. For expression of the differential movement of strontium and

TABLE II. % of Total Calcium Intake by Type of Food during 10-Day Preliminary Period and 5-Day Milk-Collection Period.*

Subject	I		II		III		IV		V	
	10 days	5 days	10 days†	5 days	10 days	5 days	10 days	5 days	10 days†	5 days
Milk products	65.4	51.5	82.4	78.7	85.1	84.4	76.4	73.0	80.8	76.7
Fruits	6.8	7.5	4.2	3.3	1.8	1.9	2.8	3.5	3.8	5.7
Fruit juice	7.4	10.3	.7	1.9	.9	1.4	3.0	3.9	.7	1.5
Vegetables	11.4	19.0	5.8	7.8	5.6	6.3	7.6	11.3	7.3	7.9
Fish and meat	2.3	3.0	1.1	1.0	1.9	1.4	1.7	1.8	2.4	1.6
Eggs	5.0	4.9	1.6	3.4	2.5	2.0	4.2	4.5	3.5	3.1
Choc. bar or candy	1.7	3.9	4.0	4.0	2.2	2.7	4.3	2.0	1.2	2.9
Peanuts			.2						.3	.5

* Calculations made from g (wet) ingested, using data in *Handbook of Biological Data.* William S. Spector, W. B. Saunders Co., Philadelphia & London, 1956.

† Preliminary period was 11 days for Subjects II and V.

calcium, the terminology of Comar(2) is used;

(Strontium-Calcium Observed Ratio):

$$\mathrm{OR}_{\mathrm{milk/diet}} = \frac{\mathrm{Sr/Ca\ of\ milk}}{\mathrm{Sr/Ca\ of\ diet}}.$$

Results. The calcium contribution of each major type of food was calculated by applying percentage composition as given by Spector (10) to wet weight consumed (Table II). Except in Subject I, milk products supplied about 80% of dietary calcium. These estimations are of general interest in comparing intakes during preliminary and collection periods. The figures may also be of interest in relation to population averages for calcium contributions of various diet components.

Table III presents a summary of analytical results. Total food intake was essentially the same for each subject. Food intake of Subject I differed markedly from that of the other subjects in that (a) calcium concentration of the diet was lower, (b) total calcium intake was lower, and (c) Sr^{90}/Ca ratio in total diet was higher. These differences were probably a result of the lower milk consumption of Subject I as compared with that of the others.

From about 3100 to 7200 g of milk were secreted during the 5-day period with Subject I being one of the highest producers. As expected, percentage of calcium in the milk fell in the narrow range of 0.023 to 0.028 for all subjects.

Perhaps the most important values are those for the Observed Ratio from diet to milk. OR values for Subjects II, III, IV, and V fell within a range of 0.085 to 0.132 with a mean value of 0.10; these values are in close agreement with those previously observed for goats and cows(4,6,7).

Subject I showed a markedly low value of 0.028. It is of interest to consider possible explanations for this low value. Subject I was consuming less than 1 g of calcium per day, yet secreting a relatively large amount (about 0.33 g) in her milk; it seems likely that skeletal stores, with a lower Sr^{90}/Ca ratio than the diet, were being mobilized for secretion into milk (note high Sr^{90}/Ca ratio of diet of Subject I). If this were so, then the procedure would inherently give an underestimate of true OR value. Since complete calcium and strontium balances were not run, this conclusion cannot be made unequivocally. An alternative explanation could be that comparative secretion of Sr into milk was lower for Subject I. Percentage of ingested Sr^{90} secreted/kg of milk by Subject I (0.13%, line 16) was lower by a factor of almost 3 than for Subjects II,

TABLE III. Analytical Results on Food Consumed and Milk Secreted during Collection Period by 5 Mothers.

Subject	I	II	III	IV	V
		Food eaten			
1. Total wt eaten (g)	14,780	13,800	15,390	12,380	13,220
2. ” ash (g)	148.29	117.55	121.89	125.23	115.49
3. Ca as % of total wt	.03	.06	.07	.06	.05
4. g Ca in 5-day sample	4.97	8.65	10.41	7.35	7.23
5. μμc Sr^{90} in 5-day sample	150.1 ± 4.3*	127.9 ± 4.0	126.6 ± 3.4	138.9 ± 7.8	106.0 ± 1.7
6. μμc Sr^{90}/g Ca	30.20 ± .76	14.79 ± .09	12.16 ± .25	18.90 ± 1.06	14.66 ± .99
		Milk secreted			
7. Total wt (g)	7146	3129	4246	4069	7233
8. ” vol (ml)	6930	3030	4140	3940	7000
9. ” ash (g)	14.22	6.27	9.54	8.92	14.95
10. Ca as % of total wt	.023	.023	.023	.027	.028
11. μμc Sr^{90}/kg of milk	.19 ± .05†	.44 ± .16	.23 ± .04	.50 ± .10	.36 ± .06
12. g Ca in 5-day sample	1.640	.709	.985	1.088	2.018
13. μμc Sr^{90} in 5-day sample	1.36 ± .38	1.38 ± .51	1.01 ± .17	2.04 ± .40	2.59 ± .46
14. μμc Sr^{90}/g Ca	.83 ± .23	1.95 ± .72	1.03 ± .16	1.88 ± .37	1.28 ± .22
		Relations between food eaten and milk secreted			
15. $OR_{\mathrm{milk/diet}}$‡	.028	.132	.085	.100	.087
16. % of ingested Sr^{90} secreted/kg milk	.13	.34	.18	.36	.34
17. Sr^{90} in milk as % of ingested Sr^{90}	.91	1.08	.80	1.47	2.44
18. Ca in milk as % of ingested Ca	32.99	8.20	9.46	14.80	27.91

* Error term is one stand. dev. from the mean of duplicate analyses.

† ” ” ” ” ” ” due to counting.

‡ $OR_{\mathrm{milk/diet}} = \dfrac{\mathrm{Sr^{90}/Ca\ of\ milk}}{\mathrm{Sr^{90}/Ca\ of\ diet}}$.

IV, and V (0.34-0.36%); however, a lowered Sr^{90} secretion (0.13 and 0.18%, respectively) could not have accounted for the difference in OR values between Subjects I and III. On the whole, mobilization of calcium stores appears to be the more likely explanation, but other possibilities cannot be ruled out. Percentage of ingested Sr^{90} secreted in total milk ranged from 0.8 to 2.4 (Table III, line 17). Subjects I, II, and III had about the same value. This again would indicate that a decreased Sr^{90} secretion into milk could not alone have accounted for the abnormally low OR value of Subject I.

Discussion. These data can be used also to estimate $OR_{\mathrm{body/diet}}$ values. It has been shown that for the cow and goat, $OR_{\mathrm{milk/diet}}$ averages about 0.4 times $OR_{\mathrm{blood/diet}}$(4). If we assume the same factor for human beings, one can calculate an average $OR_{\mathrm{blood/diet}}$ of about 0.25 for Subjects II to V; this value would be very close to the $OR_{\mathrm{bone/diet}}$. The value of $OR_{\mathrm{bone/diet}}$ of 0.25 is in general agreement with values estimated by other methods(3,7,11).

Some rather general statements can be made about expected levels of Sr^{90} in the infant as related to diet. It is anticipated that Sr^{90}/Ca ratio in the newborn would be about one-half Sr^{90}/Ca ratio of the plasma and therefore about ⅛ that of the diet of the mother during the period when the bone of the fetus was being formed; this arises in part from the preferential movement across the placenta of calcium as compared to strontium (5). If the newborn is to be breast fed, then the Sr^{90}/Ca ratio in the mother's milk would be about 1/10 of that in the mother's diet. On the other hand, if the newborn is to be given a formula based on cow's milk, then Sr^{90}/Ca ratio in the infant's diet would probably be close to that of the mother's diet. Unfortunately, we do not yet have any direct observation of the relationships between dietary values and Sr^{90}/Ca of the bone being formed in infants; on the basis of general knowledge, $OR_{\mathrm{bone/diet}}$

in the infant would probably be greater than 0.25 and less than 1. These relationships can then be used, within the limitations mentioned, to estimate radiostrontium levels in young infants from knowledge of their diet or of the mother's diet during breast-feeding.

Summary. 1. In 4 out of 5 normal healthy women Sr^{90}/Ca ratio in milk ranged from 0.085 to 0.13 of diet with average 0.10. These values are essentially the same as reported for lactating animals. The value for the other subject was 0.028, and it is considered most likely that this was a result of relatively low calcium intake (about 1 g/day), which probably led to marked negative calcium balance and thus invalidated the procedure. 2. If we assume relationship between Sr/Ca ratio in blood and milk is the same for the human as for cow and goat, it can be calculated that Sr/Ca ratio in these subjects would be about 0.25 that in diet consumed. This value is in agreement with other values obtained for man by methods based on other assumptions.

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Effect of Dilantin Sodium on Growth of Human Fibroblast-Like Cell Cultures.* (25778)

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Fibrous hyperplasia of the gingiva occurring as an untoward reaction following systemic administration of sodium diphenylhydantoinate (Dilantin Sodium) in treatment of epilepsy is a well recognized clinical phenomenon. Microscopic examination of this hyperplastic tissue characteristically shows increased fibroblastic activity with pronounced formation of new collagen fibers. Shapiro(1) has reported that administration of Dilantin to non-epileptic patients increased rate of healing of experimentally induced gingival wounds through increased connective tissue activity as well as accelerated epithelialization. In support of this clinical finding, Shafer *et al.*(2) noted that administration of Dilantin Sodium to rats produced a dramatic increase in tensile strength of healing skin wounds. They suggested that this was probably a function of increased collagenization, the same reaction which occurs in the gingival hyperplasia of patients receiving this drug. In an attempt to learn more of the mechanism for both the gingival hyperplasia and the apparent stimulation of wound healing, a study has been carried out testing the effect of Dilantin Sodium on rate of growth of human fibroblast-like cells in cell culture.

Materials and methods. The strain of fibroblast-like cells (designated as E.D.) used in these studies was originally isolated from adult human gingiva by Dr. Donald E. Flieder, St. Louis Univ. School of Dentistry and was courteously supplied by him. At the time of ini-

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TABLE I. Effect of Various Concentrations of Dilantin Sodium on Fibroblast-Like Cell Growth.

	Mean cell count (per ml of medium) $\times 10^5$	
	Initial	5 days
Control (with NaOH added equivalent to that in experimental tubes)	1.02	2.78
Control with NaCl (NaOH added + equivalent NaCl to supply Na^+ present from 200 μg Dilantin Sodium)	1.00	2.95
Dilantin (20 μg/ml of initial solution)	1.02	3.93
" (200 *Idem*)	1.00	5.90
" (1000 ")	1.03	All cells dead
" (10,000 ")	1.01	*Idem*

tial studies in our laboratory, this strain of cells was in its 17th transfer and was grown on a nutrient medium consisting of: 30% calf serum, 9% Earle's balanced salt solution (BSS) (10x conc.), 12% Eagle's basal medium (BM) (3) and triple distilled water with 100 units of penicillin, 200 μg of streptomycin and 50 units of Mycostatin (Squibb) per ml of medium. The resulting medium was adjusted to pH 7.0 with sodium bicarbonate. In the repetitive studies (I-VII,), (Table II) actively growing cultures of fibroblast-like cells were trypsinized (0.25% trypsin) for 15 minutes at 37°C, centrifuged at low speed for 20 minutes, washed in single strength Earle's BSS and centrifuged once more. The cells were then resuspended in the nutrient medium and divided into 2 bottles of 80 ml each. To one bottle was added 2 ml of Dilantin Sodium (200 μg per ml of solution) dissolved with sodium hydroxide (0.2 ml of a 1% solution per 100 ml) while to the other batch was added 2 ml only of sodium hydroxide of the same concentration. The suitable concentration of Dilantin Sodium was determined by a pilot study with results as shown in Table I. In all studies in Table II except I and II, the cells were exposed to the drug for 20 minutes at room temperature (22-23°C) centrifuged, washed and resuspended in fresh medium containing Dilantin Sodium before dispensing into the culture tubes. In studies I and II, this step of prior exposure to the drug was omitted. In study VII, concentration of the calf serum was lowered to 20% and Eagle's BM to 6% in an attempt to decrease growth rate of the cells and thereby permit possible magnification of the Dilantin effect. After obtaining viable cell counts of control and experimental suspensions and adjusting initial pH in both suspensions to pH 7.0, 4 ml portions of each of the control and experimental suspensions were dispensed into a series of tissue culture tubes and the tubes incubated at 37°C in a stationary position. At the various periods indicated in the tables, cell contents of 4 tubes picked at random from each group were suspended by addition of 4 ml of 0.25% trypsin to each and viable cells counted in a hemocytometer after staining with trypan blue (0.5%). Each value expressed in Tables II and III is the average count on 4 tubes with occasional exceptions. The effect of Dilantin Sodium was also tested on the growth of HeLa cells by an identical procedure, except that the cells were grown in 63% mixture 199, 25% lactalbumin hydrolysate (5%), 10% calf serum, and 2% sodium bicarbonate (2.8% solution) plus the usual concentration of antibiotics. In addition, the effect of Dilantin Sodium was tested on growth rate of cells isolated from a fibrosarcoma induced in a rat and courteously supplied by Dr. David F. Mitchell, Indiana Univ. At time of testing with Dilantin Sodium, the tumor was in its 17th transfer. Growth medium consisted of 5% calf serum, 7.5% Eagle's basal medium (3×), 10% lactalbumin hydrolysate (5%), 5% whole egg ultrafiltrate[†] and antibiotics.

Results. These studies (Table II) indicate that Dilantin Sodium produces a remarkable *in vitro* stimulus to growth of the fibroblast-like cell under investigation. After a period

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[†] Microbiological Assoc., Washington, D.C.

TABLE II. Effect of Dilantin Sodium on Growth of Human Fibroblast-Like Cells at Varying Time Intervals.

		Control		Dilantin		
Series	Day	Mean cell count (per ml of medium) $\times 10^5$	Stand. dev. $\times 10^4$	Mean cell count (per ml of medium) $\times 10^5$	Stand. dev. $\times 10^4$	p*
I	0	1.00	.71	.98	.75	
	1	1.30	.82	1.55	.55	$<.01$
	2	1.51	.85	1.67	.53	$.02 < p < .05$
	5	3.65	4.12	6.05	4.20	$<.01$
II	0	.98	.33	1.01	.53	
	1	1.23	.73	1.51	.82	$<.01$
	2	1.54	.82	1.74	.44	"
	5	3.48	4.43	6.10	1.82	"
	7	5.15	2.52	6.35	3.41	"
III	0	.88	.20	.87	.28	
	1	1.11	.82	1.36	.44	$<.01$
	2	1.40	.39	1.61	.46	"
	5	2.93	1.71	4.98	1.71	"
	7	4.80	2.00	5.55	3.12	$.02 < p < .05$
IV	0	1.00	.48	1.03	.53	
	1	1.07	.22	1.26	.71	$<.01$
	2	1.41	.35	1.58	.33	"
	5	2.98	1.71	4.97	.98	"
	7	4.94	1.37	5.83	1.71	"
V	0	1.01	.22	1.02	.33	
	1	1.13	.46	1.37	.26	$<.01$
	2	1.44	.44	1.57	.53	$.01 < p < .02$
	3	1.55	.41	1.69	.30	$<.01$
	4	1.70	.17	1.89	.22	"
VI	0	.98	.14	.98	.17	
	1	1.12	.32	1.39	.17	$<.01$
	2	1.46	.35	1.60	.26	"
	4	1.72	.30	1.92	.07	"
	7	5.96	.45	6.43	1.74	"
VII	0	.99	.53	.98	.64	
	1	1.01	.14	1.03	.22	.3
	4	1.99	.46	4.59	2.74	$<.01$
	6	3.12	.54	5.44	1.82	"
	7	4.00	.79	6.10	.82	"

* Calculated by Student's "t" test.

TABLE III. Effect of Dilantin Sodium on Growth of HeLa Cells at Varying Time Intervals.

		Control		Dilantin		
Series	Day	Mean cell count (per ml of medium) $\times 10^5$	Stand. dev. $\times 10^4$	Mean cell count (per ml of medium) $\times 10^5$	Stand. dev. $\times 10^4$	p*
I	0	1.05	1.01	1.04	.48	
	1	1.09	.26	1.18	.17	$<.01$
	2	1.46	.44	1.63	.14	"
II	0	1.10	.71	1.10	.44	
	1	1.12	.35	1.26	.45	$<.01$
	2	1.51	.32	1.66	.30	"
	3	1.65	.25	1.79	.26	"
	4	1.72	.26	1.92	.32	"
III	0	1.02	.32	1.00	.26	
	1	1.16	.37	1.26	.45	$.02 < p < .05$
	2	1.54	.36	1.68	.35	$<.01$
	4	1.78	.17	1.99	.14	"
	7	6.05	.32	6.86	.36	"

* Calculated by Student's "t" test.

of only one day, the treated cell cultures invariably showed increased growth in comparison with control cultures, as determined by cell counts. In nearly all series, the effect on growth reached its maximum after 4 or 5 days of incubation, and in most cases, interestingly, this difference was reduced by the 7th day. Some series exhibited nearly a 2-fold increase in the cell count at the 4 to 5 day incubation period. These increases are statistically significant at levels shown in the table.

Investigation of HeLa cells revealed a similar but less remarkable increase in cell population induced by Dilantin Sodium (Table III). The fibroblasts isolated from the fibrosarcoma evidenced no reaction to the Dilantin Sodium; in fact, growth was retarded for several days.

The explanation for this apparent *in vitro* stimulation of fibroblast-like cells is not known. It is apparently caused by the diphenylhydantoin structure (or some metabolic product) rather than to the additional sodium ions present because addition of gram-equivalent amounts of sodium did not significantly stimulate growth.

Summary. Addition of sodium diphenylhydantoinate (Dilantin Sodium) to tissue culture of human fibroblast-like cells resulted in stimulation of their proliferation. A similar but more limited stimulation to proliferation occurred with HeLa cells but there was no detectable effect on cells isolated from rat fibrosarcoma.

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Gas-Liquid Chromatography of Methyl Esters of Fatty Acid from Human and Chicken Brain Lipids.* (25779)

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Purification of individual lipid fractions followed by analysis of component fatty acids involves laborious procedures while constituent highly unsaturated acids may suffer oxidative degradation(1-5). It appears beneficial if fatty acid composition patterns of total brain lipids could be surveyed simply and rapidly by gas chromatography before more detailed analyses were attempted. In the present study analyses for a number of normal saturated, unsaturated and α hydroxy fatty acids from samples of human and chicken brain lipids are described.

Methods. Preparation of fatty acids from brain lipid. Samples of human cerebrum obtained on autopsy[†] and pooled whole chicken brains were extracted with 2:1 chloroform-methanol solution according to procedure of Folch *et al.*(6). The lipid-containing solution was concentrated by evaporation of solvent under vacuum. The concentrate was poured into 4 times its volume of ice cold acetone containing 1 ml of 5% $MgCl_2$ in 94% ethanol/100 ml of acetone(7). The precipitated lipid was then filtered off under N_2, washed several times with acetone-magnesium chloride solution, dried under vacuum, and weighed. This precipitation procedure removes acetone-soluble cholesterol which, with certain exceptions, occurs in brain as free sterol only. Lipid thus obtained had a negative reaction to Liebermann-Burchard test for β-ol sterols. Dried lipid was subjected to methanolysis by refluxing 24 hours with super-dry half-saturated methanolic HCl. Methyl esters were removed from the solution by 4 extractions with diethyl ether. The combined ether ex-

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[†] Male aged 65, cause of death cerebral hemorrhage. Involved area of brain was not used for analysis.

tracts were washed free of mineral acid, dried over anhydrous sodium sulfate, and the solvent removed under vacuum. Samples of the methyl esters were hydrogenated at room temperature and atmospheric pressure using platinum oxide as catalyst. Hydrogenation was continued until there was no further absorption of hydrogen. *Oxidation of α hydroxy fatty acids.* Samples of hydrogenated free fatty acids were prepared by refluxing the hydrogenated methyl esters for 3 hours with 1N KOH, acidifying the soaps and extracting free acids with ether. The washed, dried fatty acids were subjected to oxidation procedure described by Kishmoto and Radin(5). The α hydroxy acids are selectively oxidized and thus shortened by 1 carbon atom. After oxidation, methyl esters were again prepared by refluxing fatty acids for 3 hours with half saturated methanolic HCl. The method was verified by oxidation of α hydroxystearic, α hydroxylignoceric (cerebronic acid); mixtures of α hydroxystearic, stearic, and palmitic acids and of cerebronic, lignoceric, and behenic acids. Cerebronic acid was prepared by the method of Müller(8). Gas chromatographic analysis indicated that normal acids were unaffected by oxidation; 92% of α hydroxy acids were shortened by one carbon atom. Due to overoxidation the remaining 8% of hydroxy acids were shortened by 2 carbon atoms. *Gas chromatographic analysis.*[‡] Identification peaks of the unknowns were made by comparison of retention times with known compounds and by use of internal standards. Standards available were all of even-numbered, saturated fatty acids from C^{12} through C^{24}, oleic, linoleic, arachidonic, C^{19} and C^{21} saturated fatty acids. Tentative identifications of other peaks were made by determination of the "carbon-number" as recently suggested by Woodford and van Gent(9). The carbon-numbers were obtained by plotting retention times of positively-identified straight-chain esters against their chain length on semi-logarithmic paper (and a straight line joining them is drawn). Values corresponding to retention time of any other peak can be read off the graph indicating a hypothetical chain length of a hypothetical straight-chain ester which would be eluted at this point. This figure was termed the carbon-number. As pointed out by Woodford and van Gent, this number is characteristic of a particular ester on a specific type of stationary phase and is sufficiently constant to allow comparison with runs by other authors. Carbon-numbers calculated in our study were compared with those reported by Woodford and van Gent. Our values obtained on polar DEGS column were compared with values obtained on polar maleic adipate ethylene glycol (MAE) column. From this information, together with observations made on disappearance of certain peaks after hydrogenation, we tentatively identified unknown peaks. Quantitative determinations were made on the basis of peak areas measured with a planimeter. Chromatograms of hydrogenated samples were used to determine total amounts of fatty acids of each chain length from C^{14} through C^{24}. The time for complete elution through C^{24} was 58 minutes. When untreated samples were run there was considerable shifting of retention times of major saturated fatty acids. Degree of shifting depended on number and amount of compounds eluted between 2 major saturated peaks; the shift became large between C^{20}-C^{22} and C^{22}-C^{24} peaks; this prolonged elution time through C^{24} saturated to 71 minutes. Several peaks were observed after the C^{24} saturated, but only 4 of these were used in calculations, since after this point peaks became ill-defined. Methyl esters of α hydroxystearic and α hydroxylignoceric acid were not eluted from the column under our conditions. Therefore, amounts of α hydroxy fatty acids present in samples were determined by running hydrogenated methyl esters, which had been subjected to oxidation procedure as de-

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[‡] Instrument employed was a model 90C Aerograph with thermal conductivity cells as detectors, using a Daystrom-Weston Recorder with 1 m.v. full scale sensitivity. Carrier gas was helium. Bridge current was 200 m.a. Various polar polyester-type column packings were considered for use and diethylene glycol succinate (DEGS) was chosen as giving better resolution between several of the saturated and unsaturated fatty acids of the same chain length. A 10-foot column with ¼ inch O.D. was employed, since shorter columns gave poor resolution of the longer-chain methyl esters. Temperature for all runs was 240°C.

TABLE I. Percentage Composition of Human and Chicken Brain Lipid Fatty Acids as Determined by Gas Chromatographic Analysis of Hydrogenated Methyl Esters.

	% fatty acid			
	Human*		Chicken	
Fatty acid	1	2	Lot 1 (7 chicks 4 wk old)	Lot 2 (7 chicks 6 wk old)
C^{10}-C^{14}	3.4	1.66	1.89	2.68
C^{16}	17.25	20.01	23.35	23.17
C^{18}	45.58	47.53	44.19	42.90
C^{20}	9.21	9.54	8.94	8.21
C^{22}	11.88	10.04	15.17	13.88
C^{24}	7.94	7.51	4.98	5.87
Total odd & branched chain acids	4.74	3.71	1.48	3.29

* No. 1 and 2 refer to 2 different parts of cerebrum from the same human brain.

scribed above, and measuring the increase in odd-chain acid peaks. Since these analyses were made on hydrogenated samples, this procedure gives amounts of α hydroxy acids according to their chain length only. The odd-chain α hydroxy acids which have been reported as occurring in small amounts in brain cerebrosides(4) were not considered.

Results. Chromatography of hydrogenated methyl esters gave well-defined peaks of all even-numbered esters, from C^{14} through C^{24}, and odd-numbered esters from C^{17} through C^{23}. Peaks with retention times corresponding to C^{16} and C^{18} branched chain esters were also observed, but in amounts of less than 0.5% only. In agreement with observations of others(4) further peaks corresponding to C^{25} and C^{26} esters appeared after the well-eluted C^{24} peak. However, these peaks were ill-defined and were not included in the calculations. Approximations indicate that these longer-chain acids occur in amounts between 2 and 4% in the human and less than 3% in chicken brain fatty acids. Composition of fatty acids according to their chain length is presented in Table I.

When untreated methyl esters were chromatographed, over 25 well-resolved peaks were observed. On the basis of retention data, use of standards, collection and rechromatography, many of these peaks were positively identified. Other peaks were assigned a tentative identity on the basis of their carbon numbers. Although a higher temperature and a different, but polar, column was used in our study, carbon numbers of positively identified peaks agreed well with those of Woodford and van Gent(9). Not all peaks observed represented a single component since a number of esters had coincident retention time; retention time of C^{24} tetraenoic arachidonic) was coincident with that of C^{22} saturated (behenic) acid (Table II). With exception of 4 resolved peaks beyond C^{24} saturated, which appeared to any degree on human brain ester chromatograms only, no further peaks were resolved sufficiently to justify their use in calculations. The percentage composition (Table II) therefore represents percent of total components eluted and not absolute amounts in brain fatty acids, although in most cases the values are probably close to actual quantities present. Horwitt *et al.*(1) reported over 30% palmitic acid in chick cerebella fatty acids as determined by gas chromatography. While in our study, whole brain, not cerebellum alone, was analyzed, it appears that 30% does not represent the absolute amount of palmitic acid in chick brain lipid. These investigators did not separate any acids beyond C^{22} under their conditions, while our results and those of others (4,5) indicate that there are considerable quantities of C^{24} as well as small amounts of C^{25} and C^{26} (saturated and unsaturated) fatty acids in brain tissue. Elution of these longer acids is undoubtedly one of the bigger problems involved in gas chromatographic analysis. Temperatures above 230°C were necessary for their elution from polar stationary phase columns. Furthermore, when complex mixtures of untreated esters were chromatographed, satisfactory resolutions of most esters could not be achieved with a column much less than 10 feet in length. The separating efficiencies on the 10 foot column were determined by dividing one retention time by another. Values for separation of C^{18} saturated:C^{18} monoenoic, C^{18} monoenoic:C^{18} dienoic, C^{18} dienoic:C^{18} trienoic, C^{18} trienoic:C^{20} saturated were 1.15, 1.18, 1.13, 1.17 respectively.

Percentages of α hydroxy fatty acids as determined by increase in odd-chain acids after

TABLE II. Fatty Acid Composition of Human and Chicken Brain Lipids Expressed as % of Total Fatty Acids Eluted from DEGS Column at 240°C.

			% composition			
			Human		Chicken	
Fatty acid	Carbon	No.†	1	2	Lot 1	Lot 2
Below C^{14}			.22	.27	1.47	1.7
C^{14} myristic	14.0	14.0	.44	.54	.42	.40
C^{16} branched	15.75	15.6	.44	.27	1.27	1.89
" palmitic	16.0	16.0	18.87	20.15	27.24	24.77
" monoenoic	16.55	16.4	.88	1.09	1.48	1.42
*C^{17} branched	16.72	16.7	.22	.27		
* " monoenoic	17.32	17.3	.44	.27	2.32	1.42
C^{18} stearic	18.0	18.0	25.09	25.05	27.45	28.31
" monoenoic	18.32	18.3	28.86	29.41	22.81	21.23
" dienoic	19.02	18.9	.60	.31	.85	.59
" trienoic	19.6	19.65	.53	.31	.37	.83
C^{20} arachidic	20.0	20.0	2.85	2.96	1.0	1.12
* " dienoic	20.65	20.7	.86	.75	.63	1.12
*C^{21} saturated	21.0	21.0	.25	.14		.18
*C^{20} trienoic, C^{21} monoenoic	21.42		1.11	.53	1.97	2.48
C^{22} behenic, C^{20} tetraenoic	22.0	22.0	3.69	3.40	2.93	4.19
*C^{20} pentaenoic	22.42	22.2	.56	.51	.13	.41
*C^{23} saturated	23.0	23.0	.33	.26	.13	.34
*C^{22} tetraenoic	23.35		1.78	1.96	1.41	1.92
C^{24} lignoceric, *C^{22} pentaenoic	24.0	24.0	8.71	8.61	5.89	4.89
* " monoenoic, C^{22} hexaenoic	24.32	24.3	.62	.30	.20	.77
" unsaturated	24.75 25.15 25.72		1.21 .46 1.04	.82 1.00 .87		

* For these fatty acids, identification is tentative only.

† Figures under No. are carbon-numbers reported by Woodford and van Gent for MAE column (see text).

oxidation of hydrogenated fatty acids are presented in Table III. Kishimoto and Radin (5) reported that the cerebroside from adult rat brain contains about 66% α hydroxy acids. Since most of brain hydroxy acids are probably located in the cerebrosides and this fraction constitutes 10-12% of total brain lipid, 4-6.8% hydroxy acids found in our study fall within the expected range. In agreement with these investigators for rat brain cerebrosides we found that in total fatty acids from human brain C^{24} α hydroxy fatty acid predominated. However, amounts of C^{24} acids in chicken brain were somewhat smaller (Table III). This finding may be peculiar to that species, but since the chickens in this study were still in the growing stage, more analyses on older chicks are essential before this can be assumed. In the present study more C^{18} α hydroxy acids were found in all samples than would be expected from results of Kishimoto and Radin. However, these authors reported that some of their less pure cerebrosides contained more α hydroxypalmitic and hydroxystearic acids. This observation appears to explain our findings and indicates that these shorter chain hydroxy acids occur in fractions other than cerebrosides. Amounts of α hydroxypalmitic acid we observed were very small as indicated by increases of only 0.5-0.1% in C^{15} acids after oxidation. Variations in the hydroxy acids between the 2 samples of human cerebrum are explained by the fact that sample 1 was considerably richer in white matter than was sample 2. It is

TABLE III. α-Hydroxy Fatty Acids in Human and Chicken Brain Fatty Acids.

	% in brain fatty acids*			
	Human		Chicken	
α-Hydroxy fatty acid	1	2	Lot 1	Lot 2
C^{18}	3.05	1.11	1.73	1.24
C^{20}	.15	.16	.44	.29
C^{22}	.46	.45	.56	1.16
C^{24}	2.98	4.27	1.09	1.94
Total	6.64	5.99	3.82	4.63

* Corrected for over oxidation by 8%.

planned to analyze white and gray matter separately and from different brain areas. It is the major advantage of our procedures that very small amounts of esters are required for analysis, 3 μl being sufficient for a single chromatogram, and as little as 10 μl for total analyses including hydrogenation and oxidation. Hence it is possible to analyze gray and white matter from several different brain areas from a small laboratory animal.

Summary. Gas chromatographic analysis of normal fatty acids according to chain length from C^{10} through C^{24} and even-numbered α hydroxy acids from C^{18} through C^{24} was reported for samples from human and chicken brain. Separation of 25 resolved peaks representing saturated and unsaturated normal fatty acids was achieved by chromatography of esters on a 10 foot diethylene glycol polyester succinate column. Over half of these peaks were identified, tentative identifications were given for remainder and their relative amounts in human and chicken brain fatty acids were reported.

The authors are indebted to Dr. H. P. Friedman of Carle Hosp. Clinic and Foundation, Urbana, Ill., who supplied sample of human brain, and to Evelyn Brown for technical assistance.

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Fatty Acid Pattern of Human Bile under Normal and Pathological Conditions.* (25780)

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It was found(1) with the aid of gas-liquid chromatography (GLC) that total fatty acids of human bile constitute a complex mixture of several compounds. It was also shown that purified bile lecithin contains palmitic, stearic, oleic, linoleic, and arachidonic acids, and small amounts of several other fatty acids. Fatty acids of the bile lecithin are asymmetrically distributed on the molecule and represent about 90% of total fatty acids of human bile. The lecithin of the bile may play an important role together with bile acids in holding cholesterol in solution(2,3). If lecithin has a pronounced physiological action, differences in fatty acid composition of the bile lecithin might influence such a balanced system. The purpose of this study was to investigate which fatty acids are normally present in human bile and to see how these may vary in biliary disease.

Methods. Material. Specimens of gallbladder bile and hepatic bile were collected from different cases. Table I gives clinical data. Three cases with duodenal ulcer had no signs of biliary disease. The bile was collected into methanol and stored in a refrigerator until analyzed. Fractionation and Gas-Liquid Chromatography: Total fat was extracted with chloroform:methanol according to Folch *et al.*(4). Total fatty acids were isolated after saponification and acidification, then methylated and submitted to analysis by gas-liquid chromatography as described previously(1). The lecithin from 3 different bile

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* This work is part of investigations supported by Swedish Medical Research Council.

TABLE I. Clinical Diagnosis of 13 Patients, Made by Routine Laboratory Methods, X-ray Examination and Clinical Findings during Operation.

Patient	Sex	Age	Clinical diagnosis	Gallbladder function
H.J.	♀	66	Duodenal ulcer	Normal gallbladder
U.L.	♂	47	*Idem*	*Idem*
A.B.	♀	36	”	”
M.S.	♀	38	Gallstone disease	Funct. gallbladder
V.J.	♀	53	*Idem*	*Idem*
J.L.	♀	27	”	”
E.N.	♀	62	”	Non-funct. gallbladder
H.S.	♀	50	”	*Idem*
O.F.	♂	75	Cancer of head of pancreas	Normal gallbladder
C.C.	♀		Gallstone disease—common duct stone with subsid. jaund.	Non-funct. gallbladder
H.P.	♀	76	Common duct stone—cholecystectomized before subsid. jaund.	
T.P.	♀	43	Gallstone disease	Gallbladder occlusion by stone
E.H.	♀	53	Common duct cancer	Gallbladder occlusion by tumor infiltration around cystic duct

specimens from cases with gallstone disease (one with common duct stone without jaundice) was purified by silicic acid chromatography as described earlier(1). The purified bile lecithin was hydrolyzed through the action of snake venom phospholipase A (*Crotalus adamanteus*). Fatty acids released by phospholipase A and those obtained after hydrolysis of the lysolecithin formed from the bile lecithin were methylated and subjected to GLC. Other experimental procedures used have been described(5). Fatty acids are numbered as suggested by Insull *et al.*(6). The category $C_{20\text{-}22}$ refers to a group of unsaturated fatty acids whose structure has not been determined.

Results. Specimens of bile from subjects without biliary disease and from subjects with varying biliary disease were analyzed. Content of neutral fat + free fatty acids and of phospholipids in the bladder bile specimens analyzed was in the range of 0.5 to about 1% (1,2). The detailed results of analyses of fatty acids are shown in Table II and a summary of the proportions of the 3 main groups of acids is given in Table III. There is no significant difference in composition of total fatty acids of bile between patients without biliary disease and those with gallstone disease, with or without complications. The results of these analyses also indicate the very good reproducibility of the methods used for extraction and gasliquid chromatography. There is no difference in composition between total fatty acids of bile from gallbladders and those from hepatic bile. Because of the limited cases studied (especially normals) it is, however, not possible to make definite conclusions from our data.

Analyses were also made of total fatty acids of bile specimens from 2 patients with occluded gallbladder, one case caused by gallbladder stone, with beginning resorption of bile pigments, and one case with white bile due to a common duct cancer above and around the cystic duct. In both these cases there is a clear tendency to higher concentration of heptadecanoic acid and stearic acid (Table II). Total saturated fatty acids have increased from around 35 to 58% of total fatty acids. Furthermore, concentration of linoleic acid has decreased from 25 to about 8% of total fatty acids. Thus there are significant changes in proportions between saturated, mono-saturated and polyunsaturated fatty acids (Table III).

In Table IV fatty acid composition of human gallbladder bile lecithin from 3 patients with gallstone disease is shown. The lecithin was purified by silicic acid column chromatography and hydrolyzed with phospholipase A. Fatty acids released by action of snake

TABLE II. Detailed Composition of Total Fatty Acids of Gallbladder Bile and Hepatic Bile Patients without Biliary Disease and Patients with Gallstones. Clinical data given in Table I. Values expressed as percentage of total fatty acid methyl esters.* Component fatty acids are also designated by a dual symbol giving chain length and No. of double bonds.

		Duodenal ulcer			Gallstone disease with functioning gallbladder			Gallstone disease without functioning gallbladder		Cancer of panct. head (jaund.)	Common duct stones with subsiding jaund., C.C.*		Occluding gallbladder stone	''White bile'' Common duct cancer above and around cystic duct
Saturated														
Lauric	12:0													
Myristic	14:0	.5	1.3	.7	.7	1.0	.7	.9	.6	.7	1.3	.7	4.1	4.6
Pentadecanoic	15:0	.5	.6	.7	.6	.6	.9	.8	.8	.4	.6	.4	4.0	2.7
Palmitic	16:0	30.7	27.2	28.3	23.3	23.4	43.8	33.2	26.9	32.1	27.5	27.8	26.6	24.1
Heptadecanoic	17:0	.3	.5	.5	.7	.4	.8	.6	.7	.3	.5	.7	7.7	15.2
Stearic	18:0	4.5	4.7	5.4	5.5	6.5	3.5	4.8	4.3	4.0	8.5	6.9	13.6	13.7
Monounsaturated														
Palmitoleic	16:1	3.4	7.4	6.4	10.0	8.4	5.1	5.9	6.7	7.4	2.4	5.0	3.6	3.4
Oleic	18:1	17.6	17.4	19.2	20.2	22.7	13.1	17.7	20.8	18.5	20.0	20.6	22.7	15.8
Diunsaturated														
Linoleic	18:2	29.7	22.4	25.4	27.2	29.8	26.5	25.6	27.0	26.4	27.8	28.1	10.4	6.7
Polyunsaturated														
Linolenic	18:3	.7	.9	1.1	.5	.5		.7	.6	.4	.6	.6	2.0	.6
Arachidonic	20:4	7.6	5.0	5.7	4.6	2.7	3.5	3.6	7.1	4.2	4.1	4.5	1.9	3.6
	20-22?	4.0	6.0	5.2	6.0	3.0	2.1	5.3	4.0	5.3	6.1	4.1	3.4	7.8

* C.C.—In this case hepatic bile was analyzed.

TABLE III. Composition of Principal Groups of Fatty Acids of Gallbladder Bile of Patients with Hepato-biliary Disease. Values expressed as percentage of total fatty acid methyl esters.

	% of total fatty acids		
Subject	N-saturated FA	Mono-unsaturated FA	Highly unsaturated FA
H.J.	36.5	21.0	42.0
U.L.	34.3	24.8	34.3
A.B.	35.6	25.6	37.4
M.S.	30.8	30.2	38.3
V.J.	31.9	31.1	36.0
L.L.	49.7	18.2	32.1
E.N.	40.3	23.6	35.2
H.S.	33.3	27.5	38.7
O.F.	37.5	25.9	36.3
C.C.	38.4	22.4	38.6
H.P.	36.5	25.6	37.3
T.P.	56.0	26.3	17.7
E.H.	60.3	19.2	18.7

venom phospholipase and fatty acids of the lysolecithin were determined with gas-liquid chromatography. In this way 2 different groups of fatty acids were obtained. Fatty acids freed by phospholipase A contained mainly unsaturated fatty acids and those of lysolecithin contained mainly saturated fatty acids. The predominating fatty acid of the lysolecithin was palmitic acid. According to new work of Tattrie(7) it seems very likely that saturated fatty acids of lecithin are attached to the C-1 (alpha) ester position and unsaturated fatty acids are attached to the C-2 (beta) position. The reasons for this concept have been discussed(1). The results give further support to the conclusion(1) that there are several different lecithins present in human bile.

It is also apparent that fatty acid composition is different in the 3 lecithin specimens analyzed. In subject U. O. there is an increased amount of polyunsaturated fatty acids especially belonging to the C_{20-22} group. Further work is necessary to identify these fatty acids. In subject L.T. (with a common duct stone but without jaundice) the proportion of oleic acid was increased from about 24 to 47%.

Discussion. Study of the fatty acid composition of total biliary fatty acids of patients without biliary disease and patients with uncomplicated gallstone disease showed no significant alteration which could indicate that fatty acids excreted *via* the bile were essentially different from those of normal individuals. Because of the limited number of normal cases studied definite conclusions can not be made.

There is some indication that in patients with occlusion of the cystic duct and absorption of the bile pigments the fatty acid pattern is definitely changed. There is a significant reduction in proportion of polyunsaturated fatty acids, especially linoleic acid, and a corresponding increase in proportion of saturated fatty acids, mostly in the stearic acid concentration. These changes may be due to metabolic impairment of the lipid metabolism of the liver before occlusion of the cystic duct is complete. A partial reabsorption of fatty acids from the bile or perhaps hydrogenation of unsaturated fatty acids by

TABLE IV. Fatty Acid Composition of Human Bile Lecithin from 3 Patients (U.O., L.T., and N.H.). Lecithin was isolated by silicic column chromatography and hydrolysed by phospholipase A *(Crotalus adamantus)*. Fatty acids were determined by gas-liquid chromatography. Values expressed as percentage of total fatty acid methyl esters respective fractions.

		% of total fatty acid in respective fraction of human bile lecithin					
Fatty acid		Fatty acids freed from bile lecithin by phospholipase A			Fatty acids obtained after hydrolysis of lysolecithin		
Saturated							
Lauric	12:0						
Myristic	14:0	.6	.9	.6	1.4	1.4	5.3
Pentadenoic	15:0		.6	.7	1.6	1.5	5.4
Palmitic	16:0	15.1	8.4	17.1	61.0	66.3	53.8
Heptadecanoic	17:0		.7	.7	2.7	2.1	2.7
Stearic	18:0	4.6		1.5	8.2	11.4	6.5
Monounsaturated							
Palmitoleic	16:1	4.5	8.5	6.4	6.7	9.8	7.9
Oleic	18:1	21.9	47.4	25.9	5.0	7.5	6.6
Diunsaturated							
Linoleic	18:2	31.0	32.0	32.1	3.1		1.7
Polyunsaturated							
Linoleic	18:3		1.5				
Arachidonic	20:4	1.8	5.1				
	20-22?	20.4	8.2	8.6			3.3

Component fatty acids are also designated by a dual symbol giving chain length and No. of double bonds. Sum of each column is less than 100% because trace amounts of unusual fatty acids detected were omitted from final tabulation.

bacterial enzymes must, however, also be considered.

Study of a few specimens of lecithin purified from the gallbladder bile of subjects with gallstone disease encourages a wider study of fatty acid composition of the lecithins of bile collected from subjects with various liver affections. It will be of interest to compare fatty acid composition of the various lipids of the liver with corresponding fatty acids in the bile. This is now under investigation.

Summary. 1) Total fatty acids of human gallbladder bile and hepatic bile were analyzed qualitatively and quantitatively by gas-liquid chromatography. The 3 major fatty acids in human bile are palmitic, oleic and linoleic acid, representing around 80% of total fatty acids. 2) Analysis of composition of total fatty acids in uncomplicated gallstone disease failed to reveal any abnormality in this disease. 3) In one subject with occluding gallbladder stone and beginning absorption of bile pigments, and one subject with white bile, a reduction of the proportion of polyunsaturated fatty acids and an excess in saturated fatty acids were observed. 4) The significance of differences in relative concentrations of fatty acids of lecithins in subjects with gallstone disease is discussed.

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Metabolism of Arginine by Mouse Fibroblast, Strain L.* (25781)

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It has recently been reported(1) that periodic additions of L-arginine to cultures of mouse fibroblast cells (Earle's strain L) growing in suspension obviated the need for a medium change for periods up to 10 days or more. During this time growth rate and viability were maintained at a high level. This report presents results which may explain the need for periodic addition of L-arginine for sustained growth of the L cells.

Methods. Spinner cultures of L strain cells were set up as previously described(1), using Eagle's basal medium. The bidaily additions of L-arginine-HCl, L-citrulline and L-aspartic acid were such that final concentration of the supplement was 0.2 micromole. Initial cell concentrations were approximately 2 x 10^5 cells per ml. Samples were removed daily for estimations of cellular proliferation and viability and of ammonia content of the growth medium. Free ammonia in the medium was determined as follows: The cells were removed by a short centrifugation; 1.0 ml of the supernatant fluid was placed in a 16 x 150 mm test tube fitted with an air inlet; 2.0 ml of a saturated solution of potassium carbonate and 1 drop of octyl alcohol were added; the ammonia was driven off by vigorous aeration for 30 minutes into 4 ml of 1 N sulfuric acid(2). Amount of ammonia trapped in the acid was determined by nesslerization(3). Chromatographic analysis of the reaction products was carried out with a newly available sulfonic acid type cation exchange resin paper,† which, according to Tuckerman

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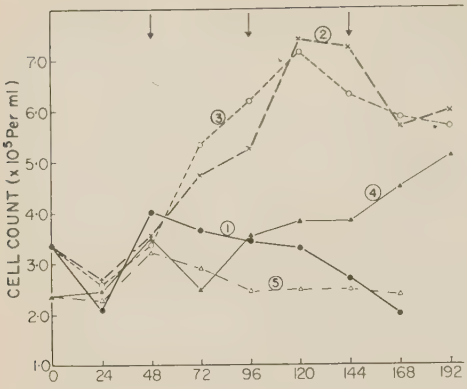
† Thanks due to Robert W. Percival, Rohm and Haas Co., Philadelphia, for generous supply of Amberlite IR-120 chromatographic paper.



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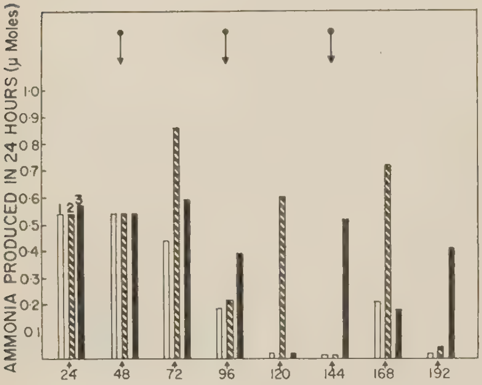

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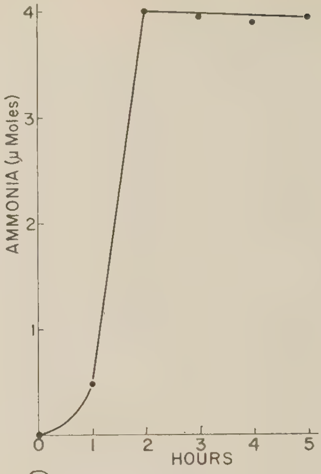

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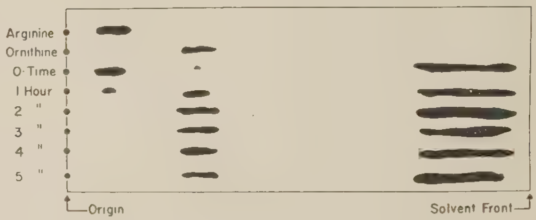

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FIG. 1. Growth of L cells in spinner culture. Curve 1, control; curve 2, arginine supplemented; curve 3, citrulline + aspartate supplemented; curve 4, citrulline supplemented; curve 5, aspartate supplemented. Vertical arrows indicate time of a supplementation.

FIG. 2. Ammonia production during growth of L cells. For significance of 1, 2, and 3, see Fig. 1. Vertical arrows indicate time of supplementation.

FIG. 3. Ammonia production by resting L cells. Amount produced by control incubation without arginine has been subtracted.

FIG. 4. Chromatogram of products produced by L cells metabolizing arginine. For methods used, see text. All monoamino acids travel as a single wide band at solvent front.

(4), allows separation of the basic amino acids by descending chromatography at room temperature with 0.2 M acetate buffer in 90 minutes. These conditions were found adequate for separating arginine and ornithine.

Results. In Fig. 1 are shown the effects of periodic addition of L-arginine and of simultaneous addition of L-citrulline and L-aspartic acid. Addition of either arginine or a mixture of citrulline and aspartic acid permitted sustained growth of the cultures. Addition of either citrulline or aspartic acid alone at this concentration was found to be not nearly as effective as the combination of the two. At twice the concentration used in these experiments, citrulline alone will sustain growth of L cells almost as well as arginine.

Amounts of ammonia formed each day are shown in Fig. 2. A large ammonia evolution occurs during the first 24 hours after an arginine addition. During the next 24-hour period, quantity of ammonia formed is the same as that in the control cultures. Amount of arginine added at each supplementation was 0.2 μM per ml and amount of additional ammonia evolved during the 24-hour period following a supplementation was 0.4-0.6 μM per ml. After supplementation with citrulline and aspartic acid, again 0.4-0.6 μM per ml of additional ammonia was liberated, but over a period of 48 hours instead of 24 hours. Urea determinations were made on the suspension culture fluids. No significant increase or decrease in urea was observed at any time.

It appeared from these observations that arginine was being catabolized during growth of L cells in the spinner culture. To determine the products of the reaction, actively multiplying L-cells were harvested from a suspended culture and placed in fresh growth medium at a concentration of 5 x 10^7 cells per ml. The culture was divided into 2 portions of 10 ml each. To one (the control) was added 0.1 ml saline, to the other 0.1 ml of a 0.2 M solution of arginine. The 2 flasks were incubated at 37° in a rotary shaker. Samples were removed every hour, placed in a boiling water bath for 10 minutes, cooled, centrifuged for 15 minutes at 25,000 x g, decanted and frozen until analyzed. Amount of ammonia formed in the flask containing arginine, in excess of that produced in the control, is shown in Fig. 3. The maximal ammonia production of 3.98 μM per ml was observed in 2 hours. Since the initial arginine concentration was 2 μM per ml, it appears that each μM of arginine was catabolized to 2 μM of ammonia. Fig. 4 is a diagrammatic representation of the paper chromatogram obtained by spotting 50 μl of the samples on the ion exchange resin paper. At zero time, only the arginine spot can be seen. At one hour, the arginine spot is still present, although reduced in size, and the ornithine spot has appeared. At 2 hours, arginine has completely disappeared and only the ornithine spot can be seen. Thus complete disappearance of arginine coincides with appearance of ornithine and 2 equivalents of ammonia.

Discussion. The growth of cells in suspended culture provides a convenient means of obtaining large numbers of cells for metabolic studies. One drawback to the procedure has been the need for periodic media changes. Thomas *et al.*(1) have reported that periodic addition of L-arginine eliminates the need for a medium change for 6-10 days. This study provides a possible explanation for the need for periodic arginine supplementation of the media. The evidence seems to indicate that L cells contain enzymes which carry out the following reactions:

(1) arginine → ornithine + 2 NH_3

(2) citrulline + aspartate → arginine

Reaction (1) seems to take place at a more rapid rate than reaction (2). Since exogenous arginine must be supplied for continued growth to occur, conversion of ornithine to citrulline must be blocked in these cells. Further studies will be required to determine which step in this conversion is not taking place.

Summary. 1. For sustained growth of mouse fibroblast cells (strain L) in suspended culture in Eagle's medium, periodic addition of arginine is essential. This requirement can be provided by arginine alone or by equimolar amounts of citrulline and aspartic acid. 2. These cells contain enzymes which catalyze the breakdown of arginine to ornithine and 2 equivalents of ammonia. 3. The requirement for periodic addition of arginine or its precursors is explained by continuous rapid breakdown of arginine observed during growth.

We acknowledge invaluable technical assistance of Thomas Orr.

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Protection by a Steroid-Spirolactone Against Certain Types of Cardiac Necroses.* (25782)

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Concurrent treatment with desoxycorticosterone and NaCl induces periarteritis-nodosa-like changes in coronary vessels, and destructive lesions of myocardium, in various laboratory animals(1,2). This showed that, in presence of excess sodium, mineralocorticoids can produce morbid structural alterations in the cardiovascular system. Recently, this concept received additional support from the finding that certain corticoids given conjointly with some sodium salts or stress can even produce acute, large, infarct-like, myocardial necroses(3). Sensitization or "conditioning" with mineralocorticoids is an indispensable prerequisite for production of all the experimental cardiac lesions just mentioned; the question arose whether an antimineralocorticoid steroid-spirolactone could offer protection against induction of the "infarctoid" large focal myocardial necroses induced by various means.

Methods. Sixty female albino rats of the Holtzman strain, with a mean initial body weight of 97 g (range: 85-118 g), were subdivided into 6 equal groups and treated as outlined in Tables I and II. 9α-fluorocortisol (F-COL)† was administered in the form of a microcrystal suspension of its acetate, at the daily dose of 750 μg in 0.2 ml of water, subcutaneously. As an antimineralocorticoid we used the steroid-spirolactone 3-(3 keto-7-thioacetoxy - 17β - hydroxy - 4 - androsten - 17 - α-yl) propanoic acid lactone, or "Aldactone"® (SC-9420)‡; this substance was given at the daily dose of 5 mg, also in the form of a microcrystal suspension, in 0.2 ml of water, subcutaneously. The sodium salts (Na_2HPO_4 or Na-acetate) were administered at the dose of 1 mM in 2 ml of water, twice daily, through a stomach tube. To expose our rats to stress, we immobilized them (by tying their legs with adhesive tape to a board in the customary manner) on the eighth day of steroid plus electrolyte treatment, during 24 hours. The first experiment (Table I) was terminated after 13 days, the second (Table II) after 9 days, by killing all surviving rats with chloroform. Immediately after autopsy, hearts and kidneys were inspected with a binocular loupe, then fixed in neutral formalin for subsequent histologic examination of sections stained with hematoxylin-phloxine and the von Kóssa calcium stain. The readings given in the Tables are the means (with standard errors) of macro- and microscopically observed organ lesions expressed in terms of an arbitrary scale of: 0 (no lesion), 1 (slightest detectable lesion), 2 (moderate lesion), and 3 (most severe lesion).

Results. The first experiment (Table I) shows that conjoint treatment with Na_2HPO_4 + F-COL (a steroid possessing both mineralo- and glucocorticoid actions) induces 100% mortality, with intense, infarct-like cardiac lesions and nephrocalcinosis, under our experimental conditions. Since most of our earlier work on production of these necroses had been performed with 2α-methyl-9α-chlorocortisol (Me-Cl-COL), which is difficult to obtain at present, it is noteworthy that the readily available F-COL can be used as a substitute in this type of experiment. The most significant result of this first series of observations was, however, that SC-9420 markedly protects against both the fatal action and the organ lesions that result from conjoint treatment with F-COL + Na_2HPO_4 (Fig. 1).

A second experiment (Table II) was then undertaken to determine whether, in F-COL-conditioned animals, the antimineralocorticoid-spirolactone also protects against development of similar lesions induced by ex-

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TABLE I. Protection by SC-9420 against Toxic Effects of F-COL + Na_2HPO_4.

Group	Treatment	Cardiac necrosis		Renal calcification		
		Severity (scale 0-3)	Incidence (%)	Severity (scale 0-3)	Incidence (%)	Mortality (%)
1	F-COL + Na_2HPO_4	2.7 ± .20	100	2.8 ± .15	100	100
2	SC-9420 + F-COL + Na_2HPO_4	.6 ± .23	50	0	0	0

TABLE II. Protection by SC-9420 against Toxic Effects of F-COL + Stress.

Group	Treatment	Cardiac necrosis		
		Severity (scale 0-3)	Incidence (%)	Mortality (%)
1	F-COL + restraint	1.5 ± .30	90	0
2	SC-9420 + F-COL + restraint	0	0	0
3	F-COL + Na-acetate + restraint	3.0 ± 0	100	50
4	SC-9420 + F-COL + Na-acetate + restraint	1.3 ± .40	60	20

posure to the stress of restraint, with or without concurrent sensitization by an Na-salt. Here, Na-acetate was employed instead of Na_2HPO_4, since concurrent treatment with F-COL + Na-acetate, unlike combined treatment with F-COL + Na_2HPO_4, rarely if ever produces any cardiac necroses in animals not exposed to stress.

SC-9420 also offers considerable protection against induction of mortality and cardiovascular lesions, under these circumstances (Table II). In absence of phosphate treatment, slight renal calcification occurred only in Group 3 (grade 0.3 ± 0.15); hence, this change is not listed in the Table.

Summary. Experiments on rats indicate that the antimineralocorticoid steroid-spirolactone 3-(3 keto-7-thioacetoxy-17β-hydroxy-4-androsten-17α-yl) propanoic acid lactone—also known as SC-9420 or "Aldactone"®—offers considerable protection against induction of acute, infarct-like, often fatal myocardial necroses normally produced by fluorocortisol plus some Na-salts and stress, alone or in combination.

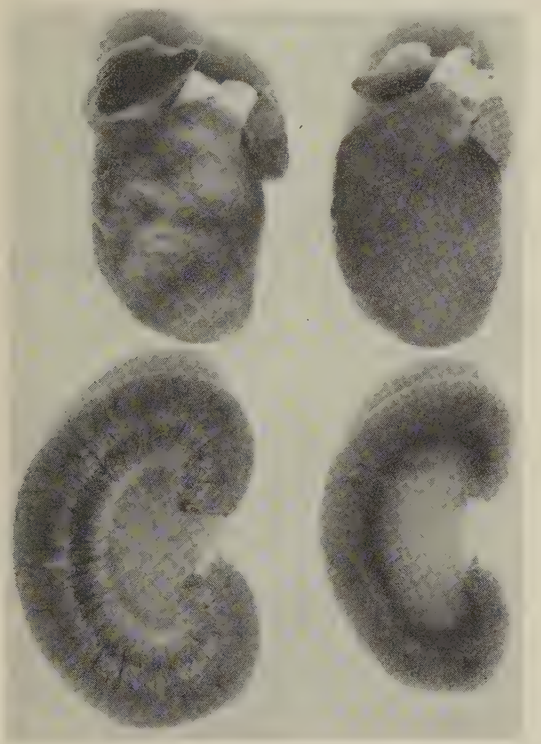

FIG. 1. Typical multiple myocardial necroses and extensive nephrocalcinosis—particularly at corticomedullary junction line—in a rat (Group 1 of first experiment) in which F-COL + Na_2HPO_4 was administered (left). Compare with the apparently normal organs of a rat (Group 2 of first experiment) treated with SC-9420 in addition to F-COL + Na_2HPO_4.

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Tremorine-Induced Rage and Its Antagonism by Atropine.* (25783)

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Tremorine (1,4 - dipyrrolidino - 2 - butyne) was reported to produce a marked parasympathetic stimulation and a sustained tremor(1, 2). Extensive use has been made of its tremorgenic effects to evaluate compounds for antiparkinsonian activity(3,4). The tremorgenic(5), analgesic(6), hypothermic(7), and stimulatory properties(8) of Tremorine were compared with those of other centrally active agents which possess these actions. We were interested in supplementing this work, most of which had been done in rodents, with Tremorine studies in the cat. During the investigation we noted that Tremorine, in addition to its previously described actions, produced a rage response which was abolished by atropine. An attempt was made to relate this response to parallel changes in cortical and subcortical electrograms and to gain an understanding of the underlying central mechanisms.

Materials and methods. Over 40 cats (2.0-3.0 kg) were used to study changes in overt behavior and brain electrical activity produced by Tremorine hydrochloride.‡ The agents were administered parenterally at following doses: Tremorine (5-20 mg/kg), atropine sulfate (2 mg/kg), and atropine methylnitrate (2 mg/kg). *Overt effects.* Tremorine (10-20 mg/kg) was studied in conscious, unrestrained animals. Atropine sulfate or atropine methylnitrate was injected before Tremorine or at peak of rage response. Postmortem observations were made, and brain and other tissues were removed for histologic study. *Electrographic studies.* These experiments were performed under anesthesia with pentobarbital (35 mg/kg), Dial-urethane (0.7 ml/kg), or while the cat was immobilized with succinylcholine chloride. A small series of encéphale isolé preparations was also studied. Spontaneous electrical activity from several cortical and subcortical areas was recorded bipolarly on a Grass polygraph using silver ball and stainless steel concentric electrodes, respectively. Doses of Tremorine slightly smaller (5-10 mg/kg) than those used to observe behavior were employed. Atropine was given either before or after the Tremorine-induced cortical activations. Blood pressure was monitored from the femoral artery during some experiments. Measures also were taken to maintain body temperature (rectal) to offset hypothermia produced by Tremorine.

Results. Overt responses and antagonism by atropine. In cats which were conscious and unrestrained different phases could be recognized during action of Tremorine (10-20 mg/kg). No signs were evident during the first hour (latency). Then there was a period of intense parasympathetic stimulation (vomiting, defecation, salivation, etc.). At this time some of the cats developed fine tremors of head and limbs. As these effects subsided, the animals became agitated and easily excited. During the next several hours the rage response which included hissing, clawing, and biting mounted in intensity (Fig. 1). The animals reacted violently to various external stimuli at which they directed their attack. This agitation, which persisted for several hours, was fatal within 24 hours if unchecked. Atropine abolished the rage response and quieted the animal within a few minutes after injection (Fig. 2). When given before Tremorine, it inhibited both the rage and parasympathetic discharge. In contrast, methylatropine at the same dose antagonized only the peripheral parasympathetic effects. At smaller, sublethal doses (less than 5 mg/kg) Tremorine did not produce rage; at higher doses (greater than 20 mg/kg) it produced ataxia, and death shortly thereafter.

Pathology. At this time only a preliminary

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‡ Furnished through courtesy of Dr. G. M. Everett, Abbott Labs.

report can be made of the pathologic effects produced by Tremorine. In enraged animals pertinent lesions were found in gastrointestinal tract, lungs, and brain. Gastric lesions were most consistent, but varied in severity from mucosal hyperemia to ulceration with hemorrhage (Fig. 4). Some of the animals evidenced hyperemia of rectal mucosa. The lungs, when involved, were congested, hemorrhagic, and often contained excessive bronchiolar secretions. Histologic sections of the brain revealed acute congestion with endothelial proliferation, edema, and various forms of neuronal degeneration in cerebrum, cerebellum, and brain stem. Necropsy of animals pretreated or treated at the height of rage with atropine did not reveal similar changes.

Cortical and subcortical electrograms. EEG changes produced by Tremorine were similar in anesthetized (Fig. 3), succinylcholine-immobilized (Fig. 5), and encéphale isolé animals and followed a comparable time sequence. The first EEG effects appeared shortly after onset of salivation (parasympathetic discharge), usually an hour after injection. Quite consistently, the records showed diffuse cortical activation which persisted during entire experimental period (Fig. 5). Onset of activation was particularly apparent when pentobarbital spindling was converted to low amplitude, high frequency activity (Fig. 3). In one of the posterior cortical leads (Fig. 5), a spontaneously occurring spike-like potential similarly disappeared during activation. Atropine consistently terminated EEG arousal and restored the control or spindling (Fig. 3) pattern within 15 minutes. Supplementary doses of pentobarbital further increased frequency of spindling.

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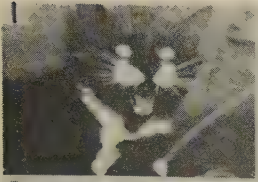

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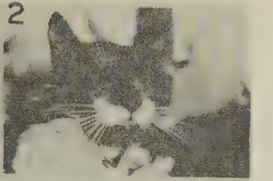

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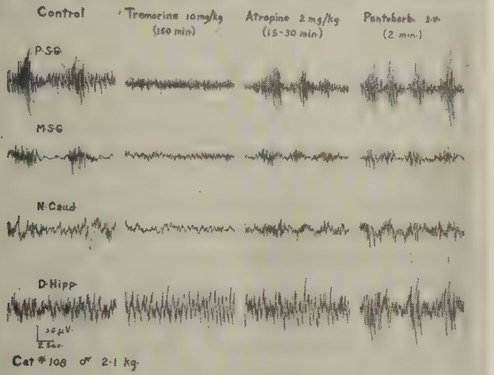

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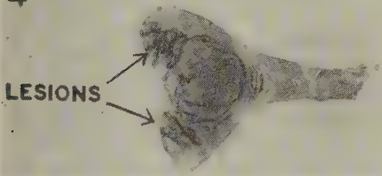

FIG. 1-4. Actions of Tremorine in cat and their antagonism by atropine. 1. Rage response. 2. Antagonism of rage by atropine. 3. Cortical and subcortical electrical changes in cat anesthetized with pentobarbital. Leads: P.S.G.—posterior suprasylvian gyrus; M.S.G.—middle suprasylvian gyrus; N. Caud.—caudate nucleus; D. Hipp.—dorsal hippocampus. 4. Gastric lesions. Time after administration of the agent indicated in parentheses.

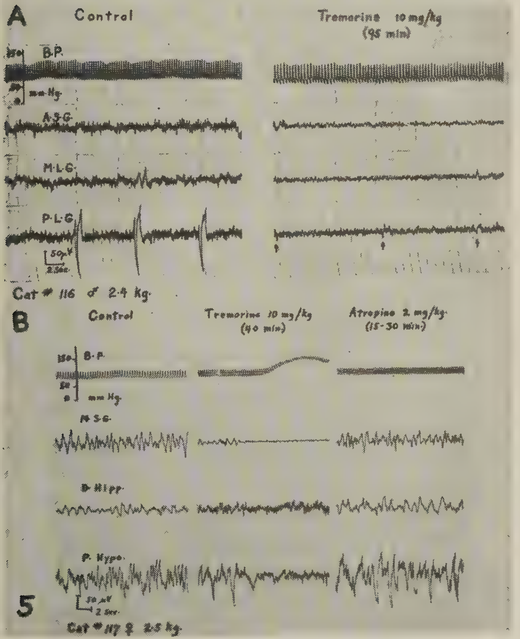

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FIG. 5. Electrographic changes produced by Tremorine in succinylcholine-immobilized cats. A. Diffuse cortical activation. B. Increased brain activity accompanied by phasic rise in blood pressure. Leads: B.P.—femoral artery blood pressure; A.S.G.—anterior suprasylvian gyrus; M.L.G.—middle lateral gyrus; P.L.G.—posterior lateral gyrus; M.S.G.—middle suprasylvian gyrus; D. Hipp.—dorsal hippocampus; P.Hypo.—posterior hypothalamus. Time after administration of agent indicated in parentheses.

None of the subcortical areas surveyed were as consistently involved as the cortex. Of the former, the posterior hypothalamic nuclei and dorsal hippocampus most often exhibited increased electrical activity (Fig. 5B). Caudate, anterior hypothalamic, and thalamic intralaminar nuclei seemed least affected. In animals anesthetized with pentobarbital Tremorine produced a steady drop in blood pressure which could be readily offset by small doses of methylatropine. Abrupt and phasic rises in blood pressure occurred in some of the succinylcholine preparations (Fig. 5B). These coincided with a further activation of cortex and increased activity in hippocampus and posterior hypothalamus.

Discussion. A central cholinergic mechanism appears to be involved in Tremorine-induced rage, since atropine, itself lacking a C.N.S. depressant action, rapidly abolishes the rage. This is further suggested by the atropine reversal of electrographic changes produced by Tremorine, thus paralleling the changes in behavior. The efficacy of atropine, in contrast to its quaternary derivative, methylatropine, is in agreement with the finding of P'an *et al.*(3) that tertiary congeners are much more effective against the central actions of Tremorine in mice.

Our results also support an interpretation which links the activated cortical EEG with the rage phase and identifies the reversal of this electrical pattern by atropine with subdued behavior. Kaelber and Correll(9) were prompted in their studies to relate the increased cortical activity directly to the tremorgenic actions of Tremorine.

The relationship between a central cholinergic action and the accompanying changes in EEG and behavior were variously interpreted. Evidence obtained by Bradley and Elkes(10) indicates that cortical activation *per se*, such as produced through the central cholinergic actions of physostigmine, need not be accompanied by any significant behavioral arousal. Nor is atropine reversal necessarily associated with sedation since atropine previously has been demonstrated to result in a pharmacologic dissociation of behavior and the EEG(11). It is significant in our investigation that such a dissociation with atropine is no longer evident after Tremorine since both EEG and behavior are in accord with sedation.

Perhaps more significance should be attached to the subcortical actions of Tremorine. Thus, the antagonism by atropine in our studies might be explained by its effectiveness in blocking an upward cortical activation resulting from hypothalamic stimulation(12). Some of the results suggest such a posterior hypothalamic involvement during rage. Also in accord with this was the increased electrical activity of the hypothalamus which preceded cortical activation and the phasic rises in blood pressure (autonomic manifestation of rage) in the succinylcholine-immobilized preparation. Ingram *et al.*(13) related changes in hypothalamic activity to EEG and behavior arousal. Prolonged stimulation of hypothalamic areas was also shown to produce gastric lesions(14) and might contribute to the lesions seen after Tremorine-induced rage.

The link between subcortical actions and central cholinergic mechanisms requires further study and is being investigated. Still unexplained are the latency, progressive nature, and long duration of Tremorine rage. These could result from a slow biochemical transformation of Tremorine to an active compound which produces rage and is then slowly metabolized.

Summary. In the cat Tremorine produced a directed, sustained rage and gastric lesions. Electrographic changes paralleling rage response included diffuse cortical activation and increased activity in posterior hypothalamus. The rage and electrical changes are antagonized by atropine and appear to involve a central cholinergic mechanism of subcortical origin.

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Effect of Zinc Deficiency in Hens on Hatchability and Embryonic Development.*† (25784)

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Following demonstration of zinc deficiency in chicks(1,2) and poults(3), an investigation of the effect of low zinc intake by the laying hen was undertaken in this laboratory. A preliminary study revealed that hens receiving a purified diet containing 6 ppm zinc laid eggs which hatched considerably poorer than those of hens fed a similar diet to which 96 ppm zinc had been added(4). Subsequently, Turk *et al.*(5) reported that hatchability was decreased when hens were fed a soy protein diet of low zinc content (8-9 ppm) and that the chicks which hatched were weak, feathered poorly and usually died within a few days.

Methods. Babcock strain White Leghorn pullets were housed in individual stainless steel cages and fed the basal diet described in Table I, estimated to contain 6 ppm zinc. Feed and distilled water were provided *ad libitum* in stainless steel containers. Fresh diet was prepared at 3-week intervals. Following a 6-week preliminary feeding period, 12 hens were fed the basal diet with 120 ppm zinc added as zinc chloride and 12 hens were continued on the unsupplemented basal diet. The hens were inseminated at weekly intervals with 0.1 cc of pooled semen from a group of flightless strain males which were maintained

TABLE I. Composition of Basal Ration.

	%		%
Isolated soybean protein*	21.7	Corn oil	4.6
		Antioxidant†	.02
Sucrose	61.63	Choline chloride	.15
DL-methionine	.6	Mineral mixture‡	10.45
Glycine	.5	Vitamin " §	.35

* ADM assay protein (Archer Daniels-Midland Co.) washed 3 times with distilled water and dried at approximately 55°C.

† 1,2-dihydro-6-ethoxy, 2,2,4-trimethylquinoline (Santoquin).

‡ C.P. or reagent grade, supplied (% of diet): 1.53 $CaCO_3$, 6.80 $Ca_3(PO_4)_2$, 0.80 K_2HPO_4, 0.27 KCl, 0.30 $MgSO_4 \cdot 7H_2O$, 0.65 NaCl, 0.05 $FeSO_4 \cdot 7H_2O$, 0.002 $CuSO_4 \cdot 5H_2O$, 0.03 $MnSO_4 \cdot H_2O$, 0.0002 $CoSO_4 \cdot 7H_2O$, 0.001 KI, 0.01 $AlK(SO_4)_2 \cdot 12H_2O$, 0.001 $Na_2MoO_4 \cdot 2H_2O$, 0.005 $Na_2SiO_3 \cdot 9H_2O$, 0.001 H_3BO_3, 0.00007 Na_2SeO_3.

§ Supplied/100 g of diet: mg, 6.6 alpha-tocopheryl acetate, .003 cyanocobalamin, 1.0 menadione, 100 inositol, .5 para-aminobenzoic acid, .08 biotin, .2 folacin, 1.8 pyridoxine HCl, 10.0 niacin, 3.4 calcium pantothenate, 2.0 riboflavin, 2.0 thiamine HCl, 3.0 ascorbic acid; also 1170 I.U. vit. A and 150 I.C.U. vit. D_3.

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* Scientific Article No. A841. Contribution No. 3128 of Maryland Agric. Exp. Station (Dept. of Poultry Husbandry).

† We are indebted to C. E. Clark and W. O. Pollard for technical assistance; to S. W. Patch and K. Pfeiffer for photography. We are also indebted to Merck & Co., Inc., Rahway, N. J., for crystalline vitamins; E. I. duPont de Nemours & Co., Wilmington, Del. for DL-methionine; Distillation Products Industries, Rochester, N. Y. for d-alpha-tocopheryl acetate; Monsanto Chemical Co., St. Louis, Mo. for Santoquin; and Hoffmann-LaRoche, Inc., Nutley, N. J. for biotin.

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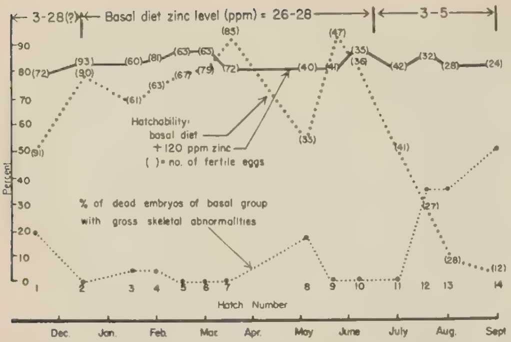


FIG. 1. Effect of zinc level of hen diet on (1) hatchability and (2) incidence of embryos with characteristic trunk and limb abnormalities. These skeletal abnormalities were never observed in embryos from hens fed the zinc-supplemented diet.

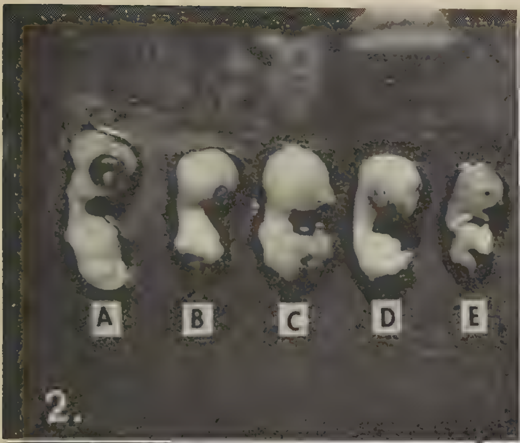
on a practical type ration containing 42 ppm zinc. Eggs were collected for 2-week periods (storage temperature 50°F) and incubated. Fertility was determined on 7th day of incubation and at this time, and periodically thereafter, all dead embryos were examined for gross anomalies. Some of the 18-19 day embryos found to be obviously malformed upon candling were examined while still living. Zinc determinations were made by the one-color dithizone procedure described by Benne(6). Certain of the terms describing embryonic malformations (anouria and ectrosomia) were taken from Ancel(7).

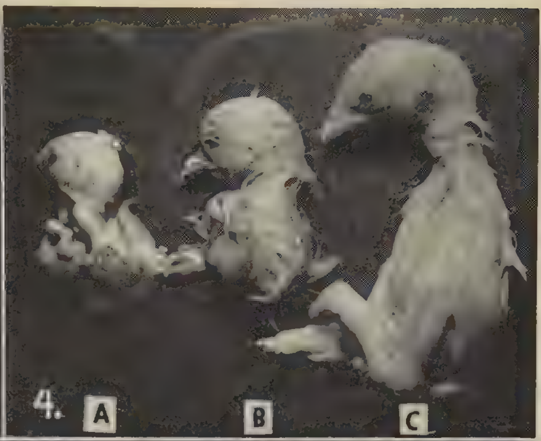
Results. The percentage of fertile eggs which hatched (hatchability) and the percentage of dead embryos which were characteristically malformed are shown in Fig. 1 for 14 hatches. Hatchability of eggs from the hens fed the zinc-supplemented diet was relatively uniform throughout the experiment, varying between 79% and 89%. No consistent type of malformation was seen in the embryos from hens receiving the supplemented diet in any of the 14 hatches.

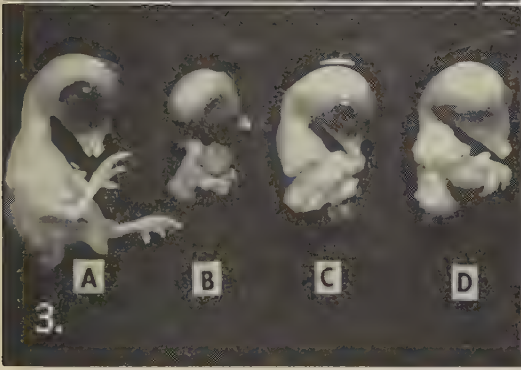
Hatchability of eggs laid by hens receiving the unsupplemented diet was erratic during the first 10 hatches, but generally lower than that of eggs from the zinc-supplemented hens. During this period (see Fig. 1) the basal diet was found by analysis to contain approximately 28 ppm zinc rather than 6 ppm as has been presumed. The contamination was eventually traced to the $CaCO_3$ being used, which, though purportedly of reagent grade, contained 1400 ppm zinc. When this product was replaced with one of suitable quality (3 ppm zinc), zinc content of basal diet was reduced to approximately 4 ppm, and hatchability rapidly decreased to zero (hatches 11-14). Decrease in hatchability was paralleled by a decrease in zinc content of the yolk of the basal group eggs from 26-38 ppm to 7.5-11 ppm(8).

In all instances, except hatch 11, whenever hatchability was lowered, gross malformations of a characteristic type were encountered in the dead embryos of the basal group eggs, incidence of these abnormalities being directly related to level of embryonic mortality (inversely related to hatchability, Fig. 1). The characterizing feature of these malformations was evidence of some degree of faulty trunk and limb development. The caudal-most part of the vertebrae was always absent (anouria) (Fig. 3) and, in some instances, a larger portion of the trunk appeared to be missing (ectrosomia) or shortened (Figs. 2,4). Dorsal curvature of the spine (lordosis) was also found (Fig. 3, embryo B). In some cases portions of the limbs (Fig. 3, embryo C), or the limbs in their entirety (ectromelia) were missing (Fig. 2 and certain embryos in Figs. 3 and 4). A common occurrence was a short leg with only 2 toes (ectrodactyly) (Fig. 3, embryo C, Fig. 4, embryo B). Occasionally, an apparent fusion of the lower limbs was observed (symmelia) (Fig. 2, embryo E).

Embryos living to develop head structures comparable to normal 6-7 day embryos frequently had abnormal brains and visceral arches, and small eyes (microphthalmia) (Fig. 3, 4), as well as ectromelia and apparent ectrosomia (Fig. 2). Exposed viscera (celosomia), though not common in 6-7 day embryos, was almost invariably associated with skeletal malformations in embryos developing beyond 7 days (Figs. 3, 4). Abnormalities of the beak and other external head structures were observed (Fig. 4). Malformed embryos living to 18-20 days were usually well-feathered but sometimes had extreme skin edema (Fig. 4, embryo A). Viscera of embryos which developed 9-19 days appeared surprisingly normal under a dissecting microscope, with heart, kidneys, gonads, lungs and intestinal tract well developed.







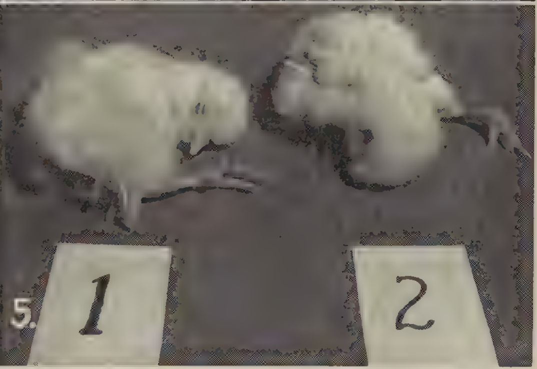


FIG. 2. A, normal chick embryo incubated 6 days. B, C, D and E, dead embryos from zinc-deficient hens developed to 6-7 days incubation. B, C, and D have ectrosomia and ectromelia. E has symmelia, lordosis, and ectromelia of wing buds. C and E have microphthalmia.

FIG. 3. A, normal chick embryo incubated 12 days. B, C, and D, dead embryos from zinc-deficient hens developed to about 12 days. Each has celosomia, anouria, and degrees of microphthalmia. B and D have complete ectromelia, while C shows ectrodactyly and micromelia. B has lordosis.

FIG. 4. C, normal chick embryo incubated 18 days. A and B, living embryos from zinc-deficient hens incubated 18 days. Both have celosomia, microphthalmia, ectromelia, and beak deformities. A has skin edema and complete ectromelia, while B has partial ectromelia, ectrodactyly, and micromelia.

FIG. 5. Chicks hatched from hens fed the unsupplemented basal diet.

In hatches 1 and 12-14, particularly, a considerable portion of the basal group chicks which started to hatch were unable to emerge from the shell. Others exhibited varying degrees of weakness after hatching, some being entirely unable to stand, and others showing a disinclination to walk and a tendency to squat (Fig. 5).

Discussion. The need for an adequate zinc intake by the breeding hen to insure normal embryonic development was confirmed in this experiment. It was indicated, furthermore, that a sufficiently restricted zinc intake can be expected to result in total loss of hatchability.

Absence of trunk and limb abnormalities in the zinc-supplemented group embryos and appearance of these anomalies in increasing numbers as hatchability of the basal group eggs decreased suggests that zinc-dependent metabolic processes are of particular importance in development of skeletal mesoderm. It is recognized, however, that histological studies might reveal equally significant, though less obvious, effects of zinc deficiency on other types of embryonic tissue.

Summary. Evidence is presented indicating that deficiency of zinc in the diet of breeding hen, results in (1) lowered hatchability (2) gross embryonic anomalies characterized by impaired skeletal development and (3) varying degrees of weakness in chicks which hatch.

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Lung Tumors in Hamsters Produced by Polyoma Virus. (25785)

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Ease of transmission of polyoma infection in mouse colonies suggests that virus is spread by way of the respiratory tract(1). Rowe *et al.* reported that mice and hamsters can be infected with polyoma virus administered intranasally, although they did not describe the tumors(2). Eddy and associates described development of fibrosarcomas and angiomatous lesions in hamsters following subcutaneous injection of polyoma virus(3). Only a few animals in their study had lung neoplasms. The present report is part of study on pathogenesis of polyoma infection in the hamster and compares virus administration routes on development of lung tumors.

Materials and methods. Tissue Cultures. Embryos were removed from pregnant mice of ICR strain on 14th or 15th day of gestation. Trypsinized cultures of embryonic cells were prepared and cultivated as described by Stewart *et al.*(4). Cells were grown in 4 oz. prescription bottles and in roller tubes. Cultures were grown in 2% calf serum in medium 199 containing penicillin and streptomycin and maintained in 1% calf serum in medium 199 by weekly changes of fluids. The strain of virus in these studies was received from Dr. Bernice Eddy. Virus was carried through 7 passages in cultures of mouse embryo cells. Harvests for passage were usually collected 14 days after inoculation. Sixth and seventh passage viruses were used. For certain tests, virus was passed through Selas .03 filters. Syrian hamsters were obtained from Lakeview Hamster Colony, Lakeview, N. J. Pregnant mothers were received 3 or 4 days before parturition. Litters of hamsters less than 24 hours old were pooled, randomized and redistributed to dams. Virus or control fluids were instilled intranasally (IN) by placing a drop over noses and forcing inhalation by closing the mouth. For subcutaneous (SC) and intraperitoneal (IP) injections, each animal received 0.1 ml of undiluted culture fluids. For intracerebral (IC) 0.02 ml was injected. Preliminary tests showed that uninfected mouse cell suspensions were not tumorigenic by inoculation routes employed.

Results. Virus injected subcutaneously resulted in gross tumors in subcutis, liver, kidney, heart and, in one instance only, in lungs. Subcutaneous tumors often occurred at multiple sites. On occasion they reached massive proportions accounting for 25% of body weight. Growth of these tumors varied and was often rapid. In one animal a tumor weighing 21 g at necropsy was palpated a week earlier as 2-3 mm nodule. The neoplasms resembled sarcomas described by Eddy *et al.*(3). Those seen in other tissues except the liver were similar in appearance. Histologically the growths had the structure of fibrosarcoma. Microscopic tumors were found in certain tissues, including brain, not detected by gross examination. Liver involvement was angiomatous with lesions occasion-

TABLE I. Production of Lung Tumors as Influenced by Route of Infection.

	No. of hamsters with			
Virus admin.	Gross tumors	Visceral tumors only	Lung & visceral tumors	Lung tumors only
Intranasally	38	9	14	15
All other routes	161	160	1	0

ally becoming quite extensive. Hemorrhages were frequently found in the peritoneal cavity.

Intracerebral route. Hamsters injected IC exhibited the same spectrum of visceral tumors as animals injected SC. In a few hamsters sarcomatous growths were observed at site of inoculation attached to periosteum on inner surface of cranium. Two animals had small angiomas apparently involving meninges covering cerebral hemisphere at inoculation site. These tumors were not studied histologically. No lung neoplasms were observed.

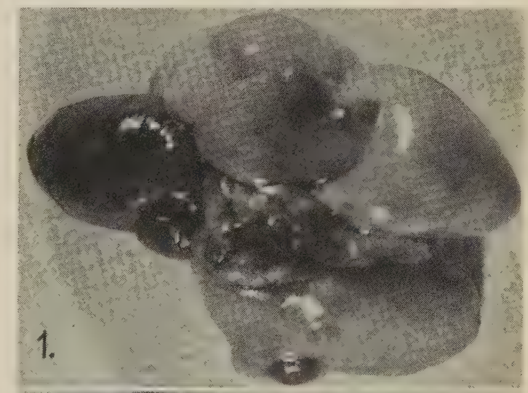
Intraperitoneal route. Animals injected IP developed only visceral tumors including angiomas of liver and sarcomata of heart and kidney. One hamster had tumorous masses on the omentum with attachment to wall of small intestine. Another had an extensive neoplasm of the ovary.

Intranasal route. Virus instilled IN induced tumor formation in tissues in the thoracic cavity. The relatively high percentage of animals developing tumors in lungs and surrounding tissues indicates that predilection was a function of method of inoculation (Table I). About one-half of animals developing lung tumors gave no evidence of other gross lesions. In the remainder there was visceral involvement in addition to pulmonary growths.

Unfiltered virus was employed for most IN studies; such preparations induced neoplasms in about 35 days. One experiment was conducted to determine if filtered fluids given IN would be tumorigenic. Two out of 19 hamsters developed lung tumors in 85 and 92 days respectively after receiving filtered virus.

Animals with lung involvement generally were thinner than controls although this difference became apparent only when animals reached maturity. Preceding death, the only sign manifested by animals with lung tumors was polypnea. Coughing was not detected. Upon necropsy of dead or sacrificed hamsters, tumors in thoracic cavity varied in extent of involvement and in gross structure (Fig. 1, 2). Some growths almost completely filled the thoracic cavity to the exclusion of virtually all normal lung tissue. Others were comprised of 1 or more nodules occupying a portion of a lobe. In several animals neoplasms involved mediastinal tissues including the heart. Grossly pulmonary growths appeared similar to sarcomas and angiomas involving viscera of hamsters injected by other routes.

Histologically, lung tumors showed 2 types of changes. One was a fibrosarcoma with typical well-differentiated spindle-shaped fibroblasts arranged in whorls and palisade groupings. The pale staining nuclei were oval-



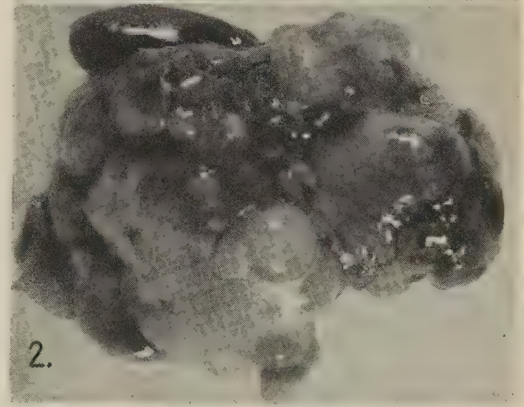


FIG. 1. Lungs from hamster sacrificed 33 days after IN infection with polyoma virus. Mediastinal and lung involvement. (Dark structure is heart.) ×15.

FIG. 2. Lungs from hamster dead 31 days after IN infection with polyoma virus. ×15.

shaped with pronounced nucleoli. Mitotic figures were rarely seen in these organized areas. Within the same tumorous nodule, focal areas of anaplasia were observed. Cells were polymorphous, exhibiting varying degrees of hyperchromatism. In anaplastic areas, a few more mitoses were seen. Lack of pattern and the wide intercellular spaces in anaplastic areas contrasted with the closely packed organized cells described above. The other main cellular change of frequent occurrence could not be identified and was presumably similar to changes described by Eddy *et al.*(3). These cells were ovoid or round with a very light staining nucleus and 1 or 2 prominent nucleoli. Cytoplasm was scanty. Cells were closely packed in rows or clusters with little stroma noted. Their identity as neoplastic cells must await further study.

An unusual amount of necrosis was seen in lung tumors. In some areas this was evidenced by extensive patches of homogenous eosinophilic matrix with accumulations of pyknotic and fragmented nuclei. In anaplastic areas, numerous necrotic cells were interspersed among actively growing tumor cells. Occasional foci or plaques of necrosis were seen in areas which otherwise had the usual neoplastic appearance. These foci were adjacent to several blood vessels, which suggests that the necrotic changes were not due to inadequate nutrition. Hemorrhage was another prominent feature of all tumors studied. Lung tissue adjacent to tumors was atelectatic. Infiltration of inflammatory cells was also noted. Occasional tongue-like processes from the tumors penetrated otherwise normal lung areas. In one area tumor cells were seen in a bronchiole.

Effects of cortisone. One experiment was conducted to test the influence of cortisone on development of tumors in polyoma-infected hamsters. Groups of randomized animals were inoculated with virus and control fluids by one of the following routes: SC, IP, IC and IN. Twenty-three days later one-half of the hamsters in each group was injected SC with 2 mg of cortisone. The experiment was terminated 15 days post-cortisone treatment, at which time surviving animals were sacrificed and examined for tumors. Observations recorded were the time that tumors were first noted, and their location. Histological studies were not made.

TABLE II. Influence of Cortisone on Tumor Development.

	Route of admin.			
	SC	IP	IC	IN
Virus	7/7†(36)‡	6/6 (36)	6/7 (34)	2/11 (38)
Virus & cort.*	6/7 (33)	6/6 (33)	7/7 (33)	9/11 (36)

* Cortisone given subcut. 23 days after virus.

† Numerator = No. with tumors. Denominator = No. inoculated.

‡ Figures in parentheses represent No. of days before tumor was noted.

Results of this experiment (Table II) suggest that cortisone enhanced development of lung tumors in the infected IN group. Thus 9 of 11 hamsters receiving cortisone had extensive pulmonary neoplasms. In untreated infected IN group, only 2 had tumors and these were visceral angiomas. There was no significant effect of cortisone on groups infected by other routes except possibly for a slight acceleration of tumor development. The spectrum of tumors was similar to that described above. All controls were negative for tumors. There was no gross difference in appearance of tumors in treated and untreated groups.

Discussion. Although considerable work on production of lung tumors in mice by chemical carcinogens has been reported, few such studies have been made in the hamster. To our knowledge the only description of virus-induced lung tumors in hamsters has been that of Eddy *et al.*(3). Growths, primarily angiomatous, were observed in lungs of several animals inoculated subcutaneously. Lesions were apparently similar to those noted in our experiments. Virus-induced lung neoplasms have also been described by Kirschstein *et al.*(5) working with fibroma virus in rabbits and squirrels. The lesions recorded were epithelial and resembled those of adenomatosis as in man and sheep(6).

Lung lesions in our study were largely those of a fibro-sarcoma. However, all tumors had areas in which there were accumulations of cells of unidentifiable type. These did not have a true malignant appearance and likely reflect the inherent nature of certain viruses to induce proliferative cellular response. Cellular necrosis observed in most of lung nodules is compatible with the fact that polyoma virus destroys cells grown in tissue culture.

Summary. Polyoma virus given intranasally produced primary lung tumors in hamsters. Some animals showed visceral involvement. Growths were largely fibrosarcomatous. Hamsters injected by other routes showed almost no lung neoplasia.

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Propagation of Trachoma Virus in Cultures of Human Tissues.* (25786)

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Although the etiological agent of trachoma was first described in 1907(1), technical limitations prevented a rapid evolution of the problem. T'ang *et al.*(2) reported on its physical characteristics and propagation in the yolk sac of chicken embryos. By omitting penicillin from the inoculum(2), virus isolation was confirmed by other workers(3-7). Subsequently, Gordon *et al.*(8) reported propagation of trachoma virus in tissue cultures of entodermal cells of chick embryos. This report describes adaptation and serial propagation of trachoma virus in tissue cultures of human origin.

Methods. The strain of trachoma virus (SA-2) was kindly provided by Dr. John C. Snyder, Harvard University(4). It had been passaged 7 times in yolk sacs of embryonated eggs. The virus was propagated by us in yolk sacs of embryonated eggs derived from a PPLO-free flock of chickens§ which were on an antibiotic-free diet. Infected yolk sacs were pooled, homogenized in a Ten Broeck grinder, and diluted 1:10 with PG medium (9). After centrifugation at 3000 rpm for 20 minutes, the aqueous supernatant, containing the virus, was stored in ampoules at dry ice temperature. Human amnion (FAM)(10) and human synovial (McCoy)(11 and personal communication) cell lines were propagated on cover slips in Leighton tubes. Tissue explants from 9-day-old chicken embryos were also used. Nutrient medium was made to volume with Mixture 199 and contained 5% heat-inactivated calf serum, 0.5% lactalbumin hydrolysate, 100 units/ml penicillin, and 0.1 mg/ml streptomycin. When confluent cell-sheets had formed and tissue explant growth was suitable, cultures were washed twice with Mixture 199 and 0.1 ml of the virus inoculum was added to each tube. Nutrient medium without antibiotics was then added to each tube (0.9 ml) and cultures were incubated at 37°C. Culture fluids were changed at 4-day intervals. Duplicate cover-slip preparations were treated with Wright's plus Giemsa stain and with acridine orange dye. The latter preparations were examined by fluorescence microscopy(12). Other cultures were frozen and thawed twice, pooled, and inoculated (0.25 ml) into the yolk sac of

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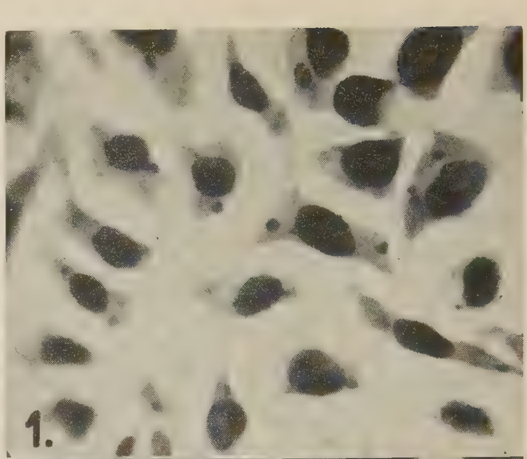
7-day embryonated eggs. At 6-8 days after inoculation or upon death of the embryo, yolk sac smears were examined for elementary bodies. Trachoma virus was passaged at 7-day intervals through 5 series of tissue cultures. Successive inoculums to tubes containing fresh tissues consisted of 0.3 ml of a pool from the previous culture. The virus was permitted to adsorb for 2 hours, then nutrient fluid, less antibiotics, was added to each tube (1.5 ml).

Results. The observations of Gordon *et al.* (8) on propagation of trachoma virus in tissues of chicken embryo explants were confirmed. Cytoplasmic inclusion bodies appeared in FAM cells within 72 hours. They occupied a vacuole and stained blue with Wright's plus Giemsa stain. Frequency of infected cells was low and maturation of the inclusion to the distinct elementary body stage was not observed. With acridine orange, the inclusion body was red-orange (RNA-staining) which coincided with the early stages of developing psittacosis virus inclusions as reported by Starr *et al.*(12).

In McCoy cells, cytoplasmic inclusions appeared within 48 hours. They were dense basophilic masses which occasionally occupied a vacuole. They increased in number and in size with age. With successive passage of the agent, inclusions became more numerous (Fig. 1). Thus, approximately 5% and 50% of cells were infected after first and the fifth passage, respectively. In acridine orange-stained preparations from fifth passage cultures, inclusions were red-orange (RNA-staining) (Fig. 2) and later matured to yellow-green (DNA-staining). Multiple stages of maturation indicated that cellular reinfection had occurred. The inclusion body was the only lesion noted.

All of the embryonated chicken eggs inoculated with tissue culture pools developed elementary bodies in the yolk sac. Virus was not infective for weanling Swiss mice inoculated *via* the intracerebral route.

Discussion. Earlier studies indicated that McCoy cells were more susceptible to infection with psittacosis virus than FAM cells. Strain SA-2 of trachoma virus showed this same relationship.



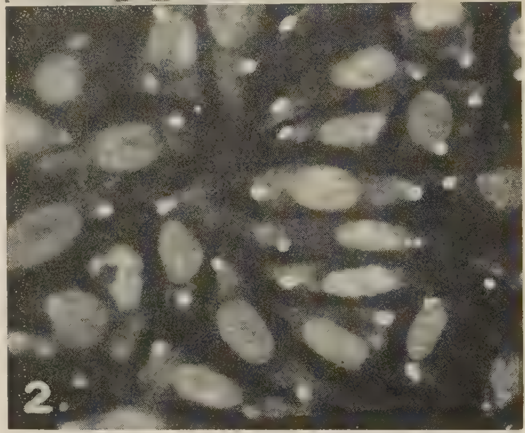


FIG. 1. McCoy cell culture infected with trachoma virus, third passage, showing inclusion bodies in cytoplasm. Wright's-Giemsa stain, ×750.

FIG. 2. McCoy cell culture infected with trachoma virus, fifth passage. With acridine orange stain and ultraviolet illumination, cytoplasmic inclusion bodies which here appear white are colored red in the original preparation, cytoplasm and nucleoli appear lighter red, and nuclei appear yellow green. ×750.

Summary. Propagation of trachoma virus (SA-2 strain) in McCoy cells was shown by 1) appearance of increased numbers of infected cells with serial passage, 2) infectivity of tissue culture fluids for embryonated chicken eggs, and 3) lack of pathogenicity for mice. The latter characteristic helps to differentiate trachoma from psittacosis viruses(2).

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Absorption and Tissue Concentrations of Vitamin B_{12} in Obese Rats. (25787)

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Bosshardt *et al.*(1) noted that growth of weanling mice on a high fat diet was markedly improved by administration of crystalline Vit. B_{12}. However, McCollum *et al.*(2) did not demonstrate any significant difference in growth of rats when Vit. B_{12} was added to diets containing different levels of fat in diet regardless of administration of Vit. B_{12}. Fox *et al.*(3) observed that the requirement for Vit. B_{12} by chicks was increased when the diet contained 22% fat, but that methionine alone could replace the increased requirement for this vitamin. They also showed that the higher Vit. B_{12} requirement was due neither to lowered absorption of dietary Vit. B_{12} nor to its subsequent storage in the liver. The involvement of Vit. B_{12} in conversion of pantothenic acid to coenzyme A has been suggested by Yacowitz *et al.*(4). In spite of these reports and the evidence presented by Ling and Chow(5) for the role of Vit. B_{12} in lipid metabolism in rats and, more recently, by Yesair *et al.*(6) in chicks, the metabolic relationship between Vit. B_{12} and lipids has not yet been established. Therefore, it is of interest to study the metabolism of this vitamin in obesity, an abnormal state of fat metabolism. The present communication presents a study of absorption and tissue concentrations of Vit. B_{12} in rats that became obese as a result of consuming a high fat diet.

Methods. Rats of the Osborne-Mendel strain were made obese by feeding *ad libitum* a diet containing 60% of fat by weight(7). They reached 1 kg of body weight in 10 to 14 months. Control rats were fed a 3% fat diet with the same composition as that of the 60% fat diet except that 57% sucrose replaced the Crisco; the control animals in earlier studies received Purina. The Vit. B_{12} concentrations in plasma and livers were determined using Skeggs' media and *Lactobacillus leichmannii* 4797 as the test organism(8). Cobalt-60 Vit. B_{12} with a specific activity of 1 $\mu c/\mu g$ was used for the absorption studies. Fifty $m\mu g$ of Co^{60}-B_{12} in 1 ml aqueous solution was administered orally through a plastic stomach tube to rats which had been fasted overnight. The feces were collected during the next 4 days, homogenized, and radioactivity was measured with a gamma-scintillation counter (9). The difference between the administered dose and amount of radioactivity recovered in the feces was considered as that which was absorbed.

Results. Vit. B_{12} levels in the plasma of obese rats were found to be higher than those in lean animals (Table I) and the differences are statistically significant. This difference is not explainable on the basis of food intake or dietary level of Vit. B_{12}, because both the 3% fat diet for control animals and the high fat diet for obese animals contained the same amount of Vit. B_{12} per gram

TABLE I. Vit. B_{12} Level of Plasma of Obese and Lean Rats.*

Group (% fat)†	Body wt (g)	Plasma B_{12} (mμg/ml)	B_{12} in diet (μg/g)
Control (3)	607	.840 ±.163‡	.200
Obese (60)	1173	1.780 ±.321	.200
Purina (10)	500	.537 ±.104	.036
Obese (60)§	895	1.260 ±.144	.200

* Five rats/group.

† % fat by wt in diet.

‡ Stand. error of mean.

§ Rats were on Purina diet for 10 mo, then placed on high fat diet for 6 mo.

(Table I). Furthermore, the obese rats consumed an average of 16 g of food per day compared to 20 g for the lean rats, therefore, intake of Vit. B_{12} was smaller. The slightly higher plasma Vit. B_{12} levels in the control group on the purified 3% fat diet as compared with those on the Purina laboratory chow may be due to the difference in Vit. B_{12} concentration in the diet. The difference in total plasma Vit. B_{12} would be even greater, since the obese rats have a greater plasma volume than the lean rat(10).

The results of Vit. B_{12} analyses in the livers of the obese rats are shown in Table II. Concentrations of Vit. B_{12} in the livers of obese animals were not significantly greater than those of the control animals. However, since the livers of obese animals were larger in size, total Vit. B_{12} content in the liver was greater in the obese than in the lean.

In view of these findings, it seemed worthwhile to investigate the intestinal absorption of Vit. B_{12} in such animals. The results of 2 experiments are presented in Table III. Radioactivity excreted in the feces following the oral dose of $Co^{60}B_{12}$ was smaller in obese rats than in lean rats and the difference is statistically significant. Thus, obese rats absorbed more of the administered Vit. B_{12}. Comparison of fecal excretion of radioactivity during the period of 4 to 7 days in the 2 groups (Study A) shows that excretion of radioactivity in this period was very small and was practically the same for the 2 groups of rats. Fecal excretion during this period may reflect the biliary and intestinal excretion of Vit. B_{12} which had already been absorbed (11).

To test whether composition of the diet rather than obese state of the animal brought about the difference in absorption rate of Vit. B_{12}, other experiments were carried out to study the effect of the fat content in the diet on absorption of Vit. B_{12}. Adult male rats were divided into 2 groups and fed the diets used in the previous experiments, containing fat at the level of 3 and 60%, respectively, for a period of 2 weeks. The rats were then tested for absorption of Co^{60}-B_{12}. The results (Table IV) indicate that there was no demonstrable difference in absorption rate between the 2 groups. Thus, it is apparent that the high fat content in the diet *per se* was not responsible for the increased absorption rate found in the obese rats.

Discussion. The published reports(1,3,4,5,6) suggest that Vit. B_{12} may play a role in lipid metabolism, although the evidence is still insufficient. The data presented herein clearly demonstrate that the rats made obese on a high fat diet containing Vit. B_{12} had greater amounts of Vit. B_{12} in the liver and higher levels of plasma Vit. B_{12} than the lean controls receiving a low fat diet although both diets contained the same amount of dietary Vit. B_{12}. Since Vit. B_{12} was determined in fasting plasma, the elevated plasma level was not a post-absorptive phenomenon, but might be metabolically characteristic in obesity. Interpretation and significance of these observations can be only speculation at the moment. The elevated Vit. B_{12} levels may not be a reflection of elevated plasma lipid levels. Total

TABLE II. Storage of Vit. B_{12} in Livers of Obese and Lean Rats.*

Group (% fat)†	Body wt (g)	Liver wt (g)	Vit. B_{12} in liver mμg/g	Vit. B_{12} in liver μg/liver
Obese (60)	1155 ± 7.5‡	25.8 ± 2.34	288 ± 44.0	7.31
" (60)§	896 ± 77.9	17.4 ± .78	234 ± 15.2	4.03
Purina (10)	515 ± 26.3	13.4 ± 1.04	248 ± 10.0	3.35

* Five rats/group. † % fat by wt in diet. ‡ Stand. error of mean.

§ Rats were on Purina diet for 10 mo, then placed on high fat diet for 6 mo.

TABLE III. Fecal Excretion of Radioactivity Following Oral Administration of 50 $m\mu g$ Co^{60}-B_{12} in Obese and Lean Rats.

	Diet	Body wt (g)	Vit. B_{12} excretion in feces ($m\mu g$) Total 4 days	 4 to 7 days	Absorbed ($m\mu g$)*
A	3% fat (6)†	643 ± 44.7	36.0 ± 2.06	1.2	14.0
	60% " (6)	1173 ± 72.4	24.9 ± 2.92	1.1	25.1
B	3% " (8)	562 ± 23.0	30.5 ± 1.33		19.5
	60% " (8)	995 ± 45.5	25.8 ± 1.34		24.2

* Calculated: 50 $m\mu g$ − amt excreted in 4 days = amt absorbed. † No. of rats.

blood lipids in obese rats have been found to be only slightly higher than in lean rats under these conditions(12).

The increased plasma concentrations of Vit. B_{12} in obesity are probably due in part to increased absorption as demonstrated in this study, although it is possible that other factors such as decreased rate of utilization and of excretion of Vit. B_{12} are also involved. The increase in absorption in obese rats is not related to body weight of the animals. Earlier work showed that normal animals weighing between 150 and 500 g absorbed practically the same amount of Vit. B_{12} when 50 $m\mu g$ was administered(13). The data in Tables III and IV also show that animals on the 3% fat diet absorbed the same amount of Vit. B_{12} even though there was a 3-fold difference in body weight of these animals. The increase in absorption does not seem to be directly related to high fat content in the diet, since the last mentioned experiment (Table IV) failed to demonstrate any immediate effect of difference in fat concentration in the diet on absorption of Vit. B_{12}. Rate of passage of Vit. B_{12} through the gastrointestinal tract has been shown to be one of the factors which determines absorption rate of this vitamin(14). The peristaltic movement of the gastrointestinal tract of the obese rats may be sluggish as are most of the physical activities in this abnormal nutritional state, thereby exposing Vit. B_{12} to the absorbing site of the intestinal mucosa longer than in normal lean rats. Although the mechanism for the increased rate of absorption is not clear, it is possible that the observed increase in Vit. B_{12} levels and absorption in obesity is a reflection of some metabolic interrelationship between this vitamin and fat.

TABLE IV. Fecal Excretion of Radioactivity Following Oral Administration of 50 $m\mu g$ Co^{60}-B_{12} to Rats on High and Low Fat Diets.

Diet	Body wt (g)	Fecal excretion ($m\mu g$)	Amt absorbed* ($m\mu g$)
3% fat (8)†	240 ± 7.8	32.4	17.6 ± 1.25
60% " (8)	232 ± 8.9	34.9	15.1 ± 1.76

* See Table III. † No. of rats.

Summary. Rats made obese by feeding high fat diet had more Vit. B_{12} in the liver and higher plasma Vit. B_{12} levels than control lean rats receiving same concentration of Vit. B_{12} in low fat diet. The obese rats absorbed more of orally administered radioactive Vit. B_{12} than the controls. The increased absorption in obese rats, however, was not directly due to high percentage of fat in the diet and would therefore be accounted for by obesity. The possible involvement of this vitamin in lipid metabolism as based on these findings has been discussed.

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Clearance of Proteins from Blood of Normal and 6-Mercaptopurine Treated Rabbits.* (25788)

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Intravenously administered protein antigens disappear from the circulation of experimental animals in 3 distinct phases(1): (1) An initial equilibration of the antigen between intra- and extra-vascular compartments takes place during the first 24-48 hours after its injection. During this phase about two-thirds of the injected antigen leaves the intravascular compartments and is distributed in the extra-vascular space (equilibration phase). (2) A logarithmic decline in circulating antigen then occurs. This phase of antigen disappearance is apparently due to catabolism of the injected protein. Circulating antibody has not been detected during this interval, which lasts a variable period of time, depending on nature of the antigen and state of sensitization of the recipient animal (exponential phase). (3) A rapid disappearance of circulating antigen begins next. This so-called immune elimination phase is due to removal of antigen-antibody complexes from the circulation, presumably by the reticulo-endothelial system(2). The exponential phase of antigen elimination has usually been ascribed to a non-immunological mechanism, but some investigators have presented data which suggest that clearance of antigen in the rabbit during this time may be due to formation of antibody, perhaps of a non-circulating type (3). This paper presents evidence that the logarithmic phase of antigen disappearance is substantially the same both in normal rabbits and in rabbits made incapable of responding to antigen by 6-mercaptopurine treatment. This means of suppressing immunity was chosen since it has been demonstrated previously that 6-mercaptopurine can abolish primary immune response of the rabbit to purified antigens(4).

Materials and methods. White, New Zealand strain rabbits weighing between 2 and 3 kg were housed in an air-conditioned animal room and fed water and Purina Rabbit Chow *ad lib*. The drinking water contained NaI, 10 mg/100 ml. Radioiodinated human serum albumin was obtained from Abbott Laboratories (RISA). Bovine gamma globulin (BGG) (Pentex, Fraction II, Lot No. 0609), rabbit gamma globulin (RGG) (Pentex, Fraction II, Lot No. 6903) and rabbit serum albumin (RSA) (Pentex, Fraction V, Lot No. 7503) were iodinated according to the method of Weigle and Dixon(5). Injections, bleedings and measurements of radioactivity were carried out as described previously(4). The T½ of the second phase of antigen disappearance was determined by graphic analysis. Fifteen animals were treated with 6-mercaptopurine according to previously described methods (4).

Results. The results are shown in Table I and Fig. 1 and 2. For each molecular species of protein the half-life of the exponential phase was shorter for heterologous material than for homologous protein. Thus, during this phase, half-life of RGG was 4.6 days, while that of BGG was 2.0 days; similarly, half-life of RSA was 5.4 days, while that of HSA was 3.4 days. Those animals in which antibody formation was inhibited by 6-mer-

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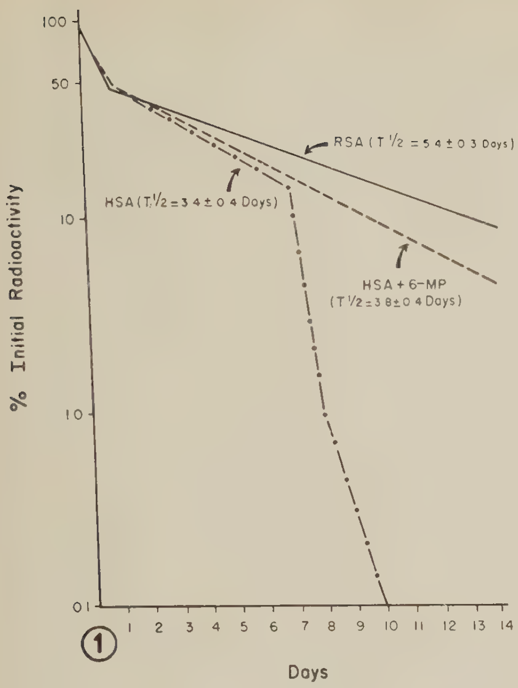

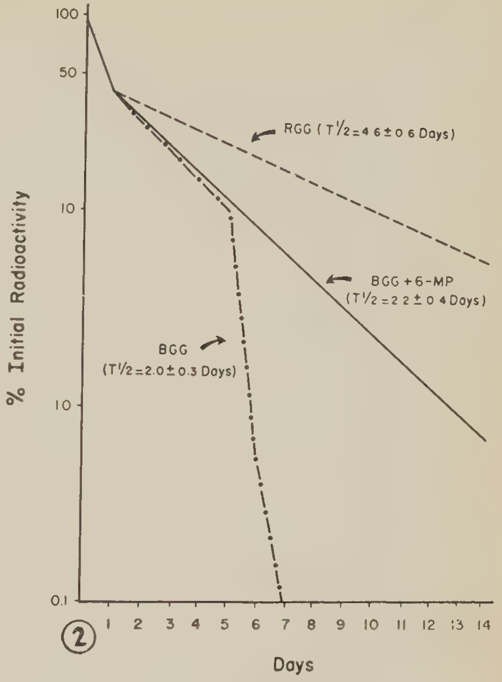

FIG. 1. Elimination of albumins from normal and 6-MP treated rabbits.
FIG. 2. Elimination of globulins from normal and 6-MP treated rabbits.

captopurine treatment cleared the antigenic protein from their circulations (after initial equilibration) at rates similar to those seen during the exponential phases of the untreated rabbits. This was also found in those rabbits unresponsive to later reinjection of the antigen (drug-induced immunological tolerance).

Discussion. Iodine^{131} labeled proteins have found wide application in investigation of a variety of immunological problems. The initial uncertainties concerning the possibility that the protein would be damaged by the iodination procedure have been resolved by development of gentle technics which make light labeling possible(6). At present, however, the use of labeled antigens yields only qualitative information concerning antibody production; *i.e.*, whether or not it occurs. It is important, therefore, to determine if antibody, either circulating or tissue fixed, can be responsible for any phase of I^{131} labeled antigen clearance other than the immune disappearance phase. Previous studies in X-irradiated rabbits and in rabbits with acquired immunological tolerance to BGG and BSA indicated that in such experimental animals an immune disappearance phase did not occur and that the half-life of antigen disappearance was the same as that of the exponential phase in normal rabbits(7). The data obtained in rabbits treated with 6-mercaptopurine are similar and support the view that the exponential disappearance phase of antigen is not due to development of antibody but to other mechanisms. Treatment with 6-mercaptopurine, furthermore, did not alter the slope of the exponential phase of antigen disappear-

TABLE I. T ½ of Circulating Proteins in Normal and 6-MP Treated Rabbits.

Protein	Rabbit test system	T ½ (days)
Rabbit albumin	Normal	5.4 ± .3
Human "	"	3.4 ± .4
Idem	6-MP treated	3.8 ± .4
"	6-MP induced "tolerance"	3.8 ± .3
Rabbit γ-globulin	Normal	4.6 ± .6
Bovine "	"	2.0 ± .3
Idem	6-MP treated	2.2 ± .4
"	6-MP induced "tolerance"	2.0 ± .2

ance indicating that this drug has no effect on non-immune clearance of foreign proteins.

It is evident that foreign gamma globulins or albumins are cleared more rapidly from the blood during the exponential phase than are homologous gamma globulins or albumins. The reasons for this phenomenon are not clear; it can be stated rather definitely that this second or catabolic phase of antigen disappearance is non-immunologic in the sense that antibodies are not found at this time. At least 2 possibilities explain these findings: (1) the same enzyme systems responsible for breakdown of homologous protein also catabolize heterologous proteins, the different rates being due to substrate differences. (2) Different enzyme systems acting at different rates catabolize homologous and heterologous proteins, even though they are of the same molecular species. These possibilities are now under study.

Summary. Rates of elimination of iodinated heterologous and homologous albumins and globulins were studied in normal and 6-mercaptopurine treated animals. The half-life of exponential phase of antigen elimination was the same in normal and in 6-mercaptopurine treated animals. These results were similar to those obtained by others in X-irradiated and tolerant rabbits and support the view that the exponential phase of antigen elimination is not due to antibody formation.

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Erythropoietin and Known Erythropoietic Stimuli.* (25789)

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It has been adequately demonstrated that hemorrhagic anemia, hypoxia, or administration of cobalt will stimulate erythropoiesis(1). Carnot(2) originally suggested the presence of an erythropoietic stimulating agent, erythropoietin, in plasma of anemic rabbits, and subsequent work has confirmed its existence (3). It was of interest to determine the effect of this erythropoietic factor derived from the plasma of anemic rabbits on erythropoietic response induced by hemorrhage, hypoxia, or chronic cobalt administration.

Materials and methods. Male and female New Zealand rabbits were made anemic by repeated injections of phenylhydrazine hydrochloride(4). Erythropoietically active plasma obtained from the anemic rabbits was boiled, filtered as described(5) and frozen until used. Male rats weighing 140 to 160 g were bled 2% of body weight by cardiac puncture under light ether anesthesia. Immediately following hemorrhage, the rats were injected intraperitoneally with an equivalent volume of erythropoietically active boiled anemic rabbit plasma or isotonic saline. Each day thereafter, 1 ml of the respective materials was administered by subcutaneous injection. Blood samples were obtained from the tail at 1, 2, 3, 4, 5, 7, and 11 days after hemorrhage for various determinations. Rats weighing 140 to 160 g at start of experiment were exposed to reduced barometric pressure (310 mm Hg) for approximately 8 hours a day, 5

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TABLE I. Effect of Injections of Boiled Anemic Rabbit Plasma Filtrate or Saline on Blood Values in Hemorrhaged Rats.

	Control		Day-1		Day-2		Day-3		Day-4		Day-5		Day-7	Day-11
Injection	Ret	Hct	Ret	Hct	Ret	Hct	Ret	Hct	Ret	Hct	Ret	Hct	Hct, %	Hct, %
	%													
Saline	2.1	44.3	11.5	32.8	7.8	31.9	12.7	32.8	16.4	34.6	16.8	35.5	40.6	44.3
Boiled filtrate	2.4	44.7	13.1	31.8	8.7	32.6	10.9	33.9	15.7	35.5	13.6	35.4	40.4	45.6

Each figure represents avg of 6-8 rats.

to 6 days a week for 4 weeks. When not in the low pressure chamber, animals were maintained at room conditions which approximated sea level pressure. One group of hypoxic rats was given 1 ml boiled rabbit plasma daily by subcutaneous injection during the 4 weeks in the low pressure chamber and for 3 weeks after removal from the chamber. The other group of hypoxic rats received 1 ml isotonic saline. Two groups of control rats for the hypoxic groups were similarly injected, but were maintained at room pressures at all times. All groups were injected for 7 weeks. Blood samples were obtained at weekly intervals. Rats were fed a diet of ground rat pellets with 0.04% $CoCl_2 \cdot 6H_2O$ added to the diet. One group received 1 ml boiled anemic rabbit plasma daily by the subcutaneous route. The other group received 1 ml isotonic saline. Two groups of rats without cobalt in the diet were similarly injected with boiled anemic rabbit plasma or saline. Blood samples were obtained at weekly intervals for reticulocyte and hematocrit percentage determinations.

Results. Standard hemorrhage. One day following hemorrhage, the hematocrit per cent of both groups decreased to minimum values (Table I). Increased erythropoiesis occurred during the first day as indicated by the increase in circulating reticulocytes, although no change in hematocrit per cent was evident until the third to fourth day after hemorrhage. A rapid increase in hematocrit per cent took place after the fifth day. By the eleventh day after hemorrhage, normal values were obtained. There appeared to be no difference in rate of recovery from a standard hemorrhage whether the animals received injection of boiled anemic rabbit plasma or isotonic saline.

Hypoxia. Exposure of rats to decreased oxygen tensions induced a marked increase in hematocrit percentages and a moderate increase in circulating reticulocytes (Table II). A plateau effect was encountered after a 2 week exposure and continued until the hypoxic stimulus was removed. During the period of increasing hematocrit percentage, there was no difference in response between the boiled anemic rabbit plasma and saline injected groups. However, animals maintained at room pressure and injected with boiled anemic rabbit plasma showed a statistically significant increase in hematocrit over those injected with saline. After the hypoxic stimu-

TABLE II. Effect of Injections of Boiled Anemic Rabbit Plasma Filtrate or Saline on Blood Values in Rats Rendered Discontinuously Hypoxic in a Low Pressure Chamber (LPC).

	Room pressure		310 mm Hg								Room pressure		
	Control		1 wk		2 wk		3 wk		4 wk		5 wk	6 wk	7 wk
Treatment	Ret	Hct	Ret	Hct	Ret	Hct	Ret	Hct	Ret	Hct	Hct	Hct	Hct
	%												
Saline LPC	1.6	44.5	3.4	51.6*	1.6	61.0*	4.8	60.3*	4.8	61.6*	51.6*	46.0	41.8
Boiled filtrate LPC	1.3	45.5	4.5	53.4*	2.6	60.5*	5.9	61.0*	6.0	59.2*	50.8*	51.2*	52.1*
Saline	1.6	44.0	2.4	44.0	4.0	44.0	3.0	41.0	2.5	44.0	43.9	45.2	44.1
Boiled filtrate	1.6	43.6	2.2	47.6*	2.4	47.3*	2.0	48.3*	2.9	50.0*	49.4*	47.2†	50.3*

Each figure represents avg of 5-6 rats.

* Significant difference from saline controls, 1% level.

† *Idem* , 5% ” .

TABLE III. Effect of Injections of Boiled Anemic Rabbit Plasma Filtrate or Saline on Blood Values in Rats Fed Cobalt.

Treatment	Initial	1 wk	2 wk	3 wk	4 wk	5 wk	6 wk	7 wk
	Hct %							
Cobalt and saline	44.4	44.8	45.7	50.8*	53.6*	53.5*	55.3*	56.0*
Cobalt and boiled filtrate	44.8	48.0*‡	50.5*‡	53.5*	52.3*	59.4*	57.0*	62.2*‡
Saline	44.0	44.0	44.0	41.0	44.0	43.9	45.2	44.1
Boiled filtrate	43.6	47.6*	47.3*	48.3*	50.0*	49.4*	47.2†	50.3†

Each figure represents avg of 5-7 rats.

* Significant difference from saline control at 1% level.

† *Idem* 5% " .

‡ " cobalt and saline group at 1% level.

lus was removed, hematocrit values of the saline injected group returned to normal. On the other hand, after return to sea level pressure the hematocrit percentages of rats which received boiled anemic plasma remained above those of the saline injected controls.

Cobalt feeding. Feeding 0.01% cobalt produced a polycythemic response in normal rats as indicated by an increase in hematocrit value (Table III). When boiled anemic rabbit plasma was administered in conjunction with the cobalt feeding an additive effect was observed.

Discussion. Boiled anemic rabbit plasma filtrate containing erythropoietin as well as anemia, hypoxia and cobalt feeding stimulate erythropoiesis. However, when prolonged erythropoietin administration is combined with either anemia or hypoxia, no additional effect is noted. Possibly the endogenous erythropoietin produced by hemorrhage or hypoxia exceeds and masks the effect of the relatively small quantity of exogenous and possibly partially denatured material injected.

The mode of action of cobalt in producing polycythemia is unknown. Unlike its effect in anemia or hypoxia, erythropoietin in boiled filtrate has an additive action in cobalt polycythemia which is evident in the early stages of combined stimulation. This result may be explained by assuming that the cobalt stimulus is suboptimal or that a different mechanism is activated by cobalt.

Summary. 1. Repeated administration of erythropoietin fails to increase the erythropoietic response to hemorrhage or hypoxia, measured by hematocrit or reticulocyte values. 2. Polycythemia is greater after simultaneous administration of cobalt and erythropoietin than after cobalt feeding alone.

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Localization of Fluorescent Antibodies to Human Growth Hormone in Human Anterior Pituitary Glands.* (25790)

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The antigenicity of human growth hormone (H.G.H.) has now been clearly established (1,2,3,4). With the advent of more highly purified preparations of H.G.H. the specificity of the corresponding antisera has been supported by studies using hemagglutination-inhibition(1,2,4) and agar gel-diffusion(3) and biological inhibition technics(3). Using the fluorescent antibody technic, Marshall (5) showed that antisera to hog ACTH localized in basophil cells of hog anterior pituitary glands. In their more highly controlled study, Cruickshank and Currie(6) were able to localize fluorescent antisera to human ACTH, TSH and gonadotropin in basophil cells of human pituitary glands. However this localization could not be inhibited with unlabelled antiserum or absorbed out by prior incubation with the specific antigen, and was therefore considered non-specific. Fluorescent antisera to human growth hormone localized in other anterior pituitary cells which were not identified. This fluorescent staining could be absorbed out by prior incubation of the antisera with H.G.H., and with ACTH and TSH as well, but could not be inhibited with unlabelled anti-H.G.H. They attributed these results to lack of purity of their hormone preparations, resulting in a lack of specificity of the corresponding antisera as demonstrated by agar gel-diffusion and precipitation-absorption technics. In this study we have attempted to examine further the specificity of our antisera to human growth hormone by investigating their localization as fluorescent conjugates in human anterior pituitaries. We hoped furthermore to demonstrate thereby the cellular site of production or storage of human growth hormone in the human pituitary gland.

Methods. Antisera to H.G.H. were prepared by immunizing rabbits with Raben H.G.H.(7). These antisera were tested for antibody titer and specificity(1). Rabbit anti-human gamma globulin (Cohn fraction II) and goat anti-rabbit gamma globulin were similarly prepared. Fluorescin isothiocyanate was prepared according to Riggs(8) and coupled to immune gamma globulin according to Marshall(9). The conjugates were absorbed with mouse liver powder prior to use (10). Pituitaries used for specificity studies were obtained at autopsy less than 2 hours post mortem. They were frozen in liquid nitrogen and stored at minus 20°C. Sections were prepared according to the method of Vasquez (11). They were not washed prior to fixing as this washes the parenchymal cells out of the gland. Pituitaries and pituitary adenomata used to demonstrate specific cellular localization were formalin-fixed paraffin-embedded blocks. Four micron sections were cut and deparaffinated in the routine manner except that they were not exposed to formaldehyde vapor. Staining of sections with the fluorescent conjugates and inhibition control studies were performed as described by Vasquez(11). All slides were mounted in buffered glycerine and studied with a Leitz fluorescence microscope unit. The formalin-fixed paraffin-embedded blocks were cut and mounted in pairs of adjacent sections. The first section of the pair was flipped over prior to being placed on the slide; the second section was mounted on the slide right side up so that the uppermost aspects of both sections were adjacent sides of the same interface and mirror images of each other. In this way we could be reasonably certain that we were looking at the same cell in both slides. The first slide was stained with the fluorescent conjugate and the second with either H and E

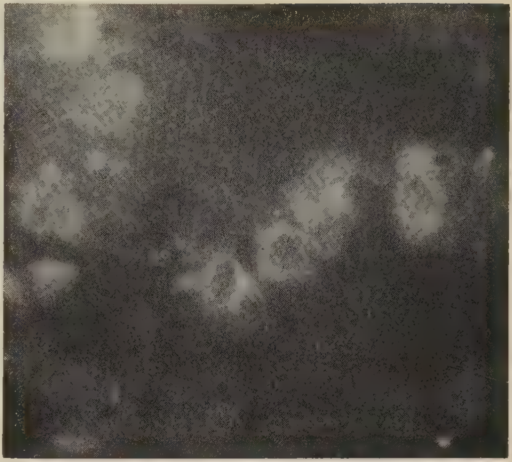

FIG. 1. Frozen section of human anterior pituitary stained with fluorescent anti-H.G.H. Note bright cytoplasm with dark nuclei. Scattered small bright spots are artifacts.

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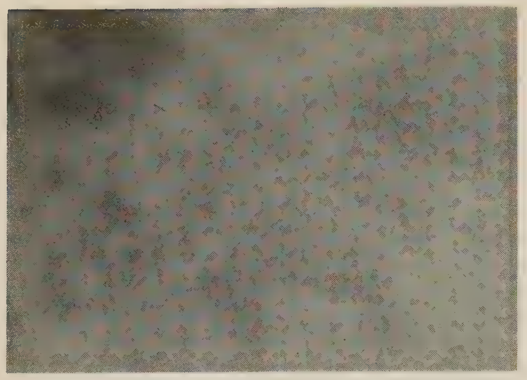

FIG. 2. Frozen section—human anterior pituitary (same as Fig. 1) stained with fluorescent anti-H.G.H. that has been absorbed with H.G.H. Note absence of specific staining.

or Aldehyde Fuchsin(12). Photomicrographs were made of identical fields. Those of the fluorescent sections only were printed using the reverse side of the transparency, so that the resultant prints would be superimposable.

Results. Frozen and formalin-fixed sections of normal human anterior pituitaries stained with fluorescent anti H.G.H. showed bright apple-green fluorescence in groups of cells and single cells (Fig. 1, 4). Unstained sections showed no fluorescence. There was no specific staining in the posterior pituitary, brain, parathyroid gland, thyroid gland and nasal polyps. Background fluorescence was higher in all of the formalin-fixed sections.

The fluorescent staining was markedly reduced in the frozen sections and completely absent in the formalin-fixed sections when stained with fluorescent anti-H.G.H. that had been previously absorbed with H.G.H. in the test tube (Fig. 2).

In the paired inhibitions studies, localization of fluorescence in anterior pituitary cells was not inhibited by prior incubation of the slide with non-fluorescent anti-human or anti-rabbit gamma globulin, but was much reduced in the corresponding slide which had been incubated with non-fluorescent anti-H.G.H. The frozen sections of human pituitaries did not stain with goat anti-rabbit gamma globulin. When stained with fluorescent anti-human gamma globulin they showed bright fluorescence in the walls of blood vessels and supporting framework but not in the parenchymal cells (Fig. 3).

Two eosinophilic adenomata, from patients with acromegaly showed specific staining in all the adenomata cells (Fig. 6). The staining was absent when stained with fluorescent anti-human growth hormone previously absorbed with human growth hormone. Chromophobe adenomata from patients with no history of acromegaly showed no fluorescence in any of the cells when stained with fluorescent anti-H.G.H. An unclassified pituitary adenoma from a patient with gross Cushings' Disease showed no specific staining of adenomata cells.

When mirror images of adjacent sides of the same interface (one stained with anti-human growth hormone, the other with the Gomori aldehyde fuchsin method) were studied, it was seen that the fluorescence was localized in most but not all eosinophil cells. It was not possible in these slides to detect differences between those eosinophils which showed fluorescence and those which did not. With very few exceptions (less than one cell per hundred cells studied) there was no fluorescence in cells other than eosinophils (Fig. 4, 5).

Discussion. Previous studies attempting to localize specific anterior pituitary hormones in pituitary glands by the fluorescent antibody technic(5,6) have been hampered by lack of purity of the hormone preparations used as antigens. With the use of a more highly purified H.G.H. preparation now available a specific antiserum can be obtained.

Our studies further indicate that these an-

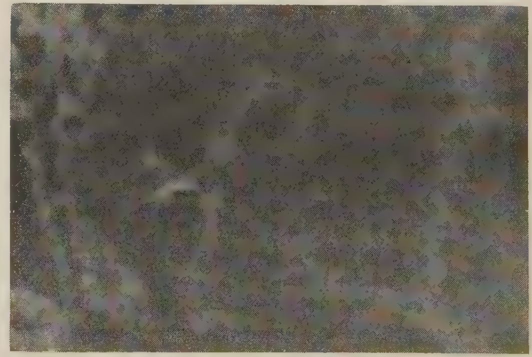

FIG. 3. Frozen section—human anterior pituitary stained with fluorescent anti-human gamma globulin. Note staining of fibrous framework and absence of staining in parenchymal cells.

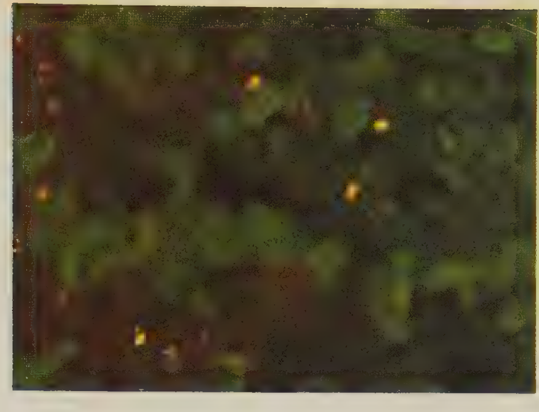

FIG. 4. Formalin-fixed section—normal human anterior pituitary stained with fluorescent anti H.G.H. Note bright green fluorescence in some cells. On comparison with Fig. 5 these cells can be seen to be acidophils.

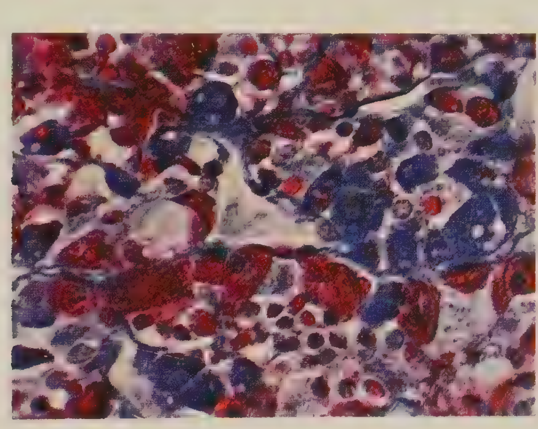

FIG. 5. Adjacent section to Fig. 4—stained with the Aldehyde-fuchsin method. Basophil and chromophilic cells correspond to non-fluorescent areas of Fig. 4.

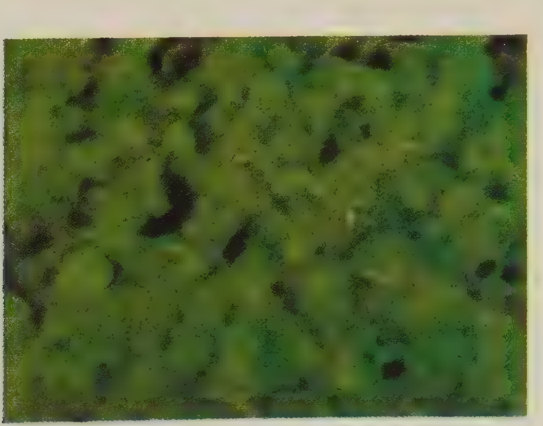

FIG. 6. Eosinophilic adenoma from a patient with acromegaly stained with fluorescent anti H.G.H. All adenoma cells are brightly stained.

tibodies react not only to human growth hormone preparations which had been used as antigen, but also with an antigen located in eosinophil cells of human anterior pituitaries and eosinophilic adenomata from patients with acromegaly. The specificity of the reaction is demonstrated by the staining of anterior pituitary cells exclusively with fluorescent anti-H.G.H. and by the following controls. This staining can be inhibited with unlabelled human growth hormone; it can be absorbed out with human growth hormone; it does not localize in tissues other than human anterior pituitary glands and eosinophilic adenomata, and fluorescent antisera of heterologous nature do not localize in the parenchymal cells of the human pituitary gland.

These experiments strongly suggest that human growth hormone is either produced or stored in most, but not all, the eosinophil cells of the human anterior pituitary. The very few exceptions to the exclusive localization of the fluorescent anti-human growth hormone in the eosinophil cells in the adjacent section studied is probably within the experimental error, but another explanation may be required. It is unlikely that the localization of fluorescence in the few basophil cells is due to a contaminating antibody or we would have expected more basophil cells to be stained.

Conclusions. Using the fluorescent antibody technic it has been shown that labelled rabbit antibodies to Raben human growth hormone localized specifically in cells of the human anterior pituitary gland. This antibody probably localizes exclusively in eosinophil cells of normal human pituitaries and in eosinophilic adenomata from patients with acromegaly.

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Distribution and Excretion of Radiofluoride in the Human.* (25791)

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During recent years there has been increased interest in fluoride metabolism particularly in regard to adoption of water fluoridation for partial prevention of dental caries(1). Because the fluoride ion is a trace element and occurs biologically in very small quantities‡ use of a radiotracer for human metabolism studies has many advantages. The only useful radioisotope of fluorine is F^{18} which is a positron emitter with a half-life of 109.7 minutes (3). The present study, utilizing radiofluoride to label 1 mg of fluoride, was undertaken to evaluate distribution and urinary excretion of fluoride ingested as a single dose by the human. One mg of fluoride was chosen because it is the quantity present in a liter of water fluoridated at 1 ppm.

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† U.S.P.H.S. post-doctoral fellow.

‡ i.e., human plasma fluoride values are of the order of 0.1-0.3 ppm(2).

Methods. Five microcuries§ of radiofluoride and 1 mg of stable fluoride as NaF contained in 250 ml of water were ingested by 2 volunteers. Each subject drank 250 ml of water at one hour and at 30 minutes before and 30 minutes after ingesting the fluoride solution (Table I). Additional water was taken over the remainder of observation period, lasting up to 5 hours, to permit collection of urine specimens at 10-15 minute intervals. Blood samples were obtained periodically by venapuncture and heparin was used as an anticoagulant (Table I). Salivary flow was stimulated by chewing paraffin and parotid gland saliva was collected by use of Lashley cups(4) placed over the orifice of each of Stenson's ducts. The saliva from the 2 glands was pooled in each sample. Radiofluoride concentrations of the fluids were determined by counting 4 ml volumes of whole blood, plasma, saliva and 10 ml volumes of urine in a well-type crystal scintillation counter. A probe type scintillation counter was used to evaluate, at frequent intervals, radiofluoride content of several body areas whose positions could be relocated. These measurements were made to indicate tissue retention of 1 mg of fluoride, labeled with F^{18}, ingested as a single dose. Owing to differences in instrumentation the activities measured over body areas cannot be directly compared with those of body fluids. All radioactivity measurements, after correction for background counts, and volume were adjusted for radioactive decay to a single point in time. Fluoride(2) in plasma and creatinine(5) and chloride(6) in plasma and urine were determined by standard methods. Renal clearances were calculated by standard procedures.‖ At the plasma fluoride levels in our studies 96-97% of plasma fluoride is freely diffusible through collodion membranes which would not pass albumin or larger molecules. Concentrations of fluoride in protein free ultrafiltrate and plasma water are equal(7) as a result of the Donnan membrane phenomenon. Plasma F^{18} concentrations were obtained from a curve delineated by the several physical measurements.

TABLE I. Determination of Renal Clearance of Radiofluoride.

Time of procedure (midpoint in min.)	Urine vol	Blood vol	Water ingestion vol
	(ml)		
–60			250
–30			250
0			250
12	105		
22		20	
29.5	360		
30			250
40		20	
40.5	135		
46	116		
60		20	
62	104		
72.5	129		
84.5	102		
97	102		
108	38		
118	34		
120		20	
129	33		
140.5	46		
151	62		
161.5	44		
173	38		
180			180
185	32		
197	18		
208	14		
225	8		
240		20	
245			180
252.5	13		
282	87		
303.5	104		
321	163		

Results. Fluoride contents of the plasma were 0.17 ppm ± 0.00 (3 samples, WDA) and 0.13 ppm ± 0.02 (5 samples, CHC). From Fig. 1 (C & D) it is seen that maximum plasma radiofluoride concentration was reached within 60 minutes and that parotid saliva radiofluoride concentrations were al-

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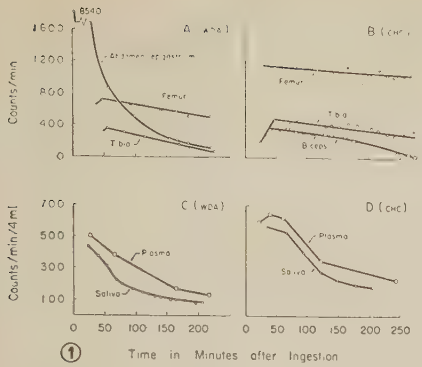
§ Radiofluoride (F^{18}) was assayed by gamma ray spectrometer comparison of annihilation gamma ray activity with that of standard Na^{22} samples obtained from Bureau of Standards, Washington, D.C.

‖ Renal clearance (ml/min) =

$$\frac{\text{Urine conc. (cts/min/4 ml) x Urine flow (ml/min)}}{\text{Plasma conc. (cts/min/4 ml)}}$$



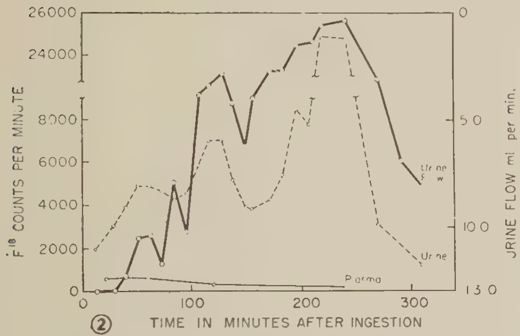


FIG. 1. A & B. Radiofluoride activity determined by portable crystal scintillation counter. C & D. Radiofluoride activity of 4 ml samples of blood and saliva.

FIG. 2. Radiofluoride content of each sample of plasma and urine corrected to a 4 ml volume is given on the left ordinate. Urine flow rate is shown on inverted right ordinate.

ways below corresponding plasma concentrations. Less than one percent of ingested activity was recovered in parotid saliva samples. Mean salivary flow rate for WDA was 0.78 ± 0.06 ml/min and for CHC was 0.37 ± 0.02 ml/min. Calculations from hematocrit values(8) of 9 samples of blood and radiofluoride concentrations of blood and corresponding plasma samples showed that 72 ± 5% of the isotope in the blood was present in the plasma. By using body weight to estimate total plasma volume it can be calculated that not more than 10% of ingested radioisotope was present in the entire plasma volume at any one time.

Fig. 1 (A &B) shows portable crystal scintillation counter observations over the indicated body areas.¶ The counts recorded over the abdominal (epigastric) area probably included the radioisotope present in liver and spine as well as that in the stomach. The initial rapid decline in these counts was undoubtedly due in large part to gastric emptying and to absorption. Rate of decline of abdominal area counts decreased with time, and after 150 minutes, was nearly equal to rate of decline of the counts over the greater trochanter of femur and tibia. This similarity in rate of change in isotope content suggests that the counts over the abdominal area recorded after 150 minutes were due mainly to radioisotope in the spine. The counts per minute measured at a right angle to the contracted biceps muscle declined at a rate similar to that measured over the femur and the tibia until after 150 minutes (Fig. 1 B). After that time biceps counts declined more rapidly than counts over the femur and tibia and at 250 minutes muscle counts were nearly zero whereas counts over the femur had decreased by only 15% from maximum value. This relative difference in retention of the isotope is due to association of fluoride with the bone mineral whereas in muscle fluoride is present only in tissue water. Furthermore, bone mineral, in contrast to muscle, contains a large amount of stable fluoride which acts as an exchange reservoir. These measurements indicate that movement of fluoride from epigastrium and uptake in the tissues occurs rapidly and that hard tissues retain their fluoride even though plasma activity during period of measurements has decreased markedly.

Renal concentration ratios (column 2 of Table II) ranged from a low of 7.8 to a high of 118. This degree of concentration is remarkable for a halogen. Radiofluoride clearance never exceeded creatinine clearance. Column 4 shows that clearance of fluoride in the human fluctuates, and increases with an increase in urine flow rate (columns 3 and 4). In particular it is to be noted (column 5) that fluoride clearances were always manyfold greater than chloride clearances. One-third of ingested radioactivity was recovered from the urine within 4 hours.

Fig. 2 summarizes in detail the measured

¶ The crystal detected the radiation through the depth of the body and within a solid angle of 120° from the aperture.

TABLE II. Renal Clearance Values.*

Subject and wt	F^{18} urine / F^{18} plasma	Urine flow, ml/min.	F^{18} clr, ml/min.	F^{18} clr / Cl clr	F^{18} clr / Creat clr	% tubular reabsorption of F^{18}
W.D.A. 80 kg	43.8	1.2	51	104	.40	60
	46.2	1.4	65	112	.39	61
	25.8	1.8	46	65	.52	48
	35.8	1.9	68	106	.40	60
	31.2	2.4	76	109	.49	51
	37.5	3.3	125	93	.47	53
	23.0	3.4	77	56	.41	59
	14.7	4.9	71	45	.41	59
	16.5	6.0	99	51	.50	50
	19.4	6.9	133	61	.40	60
	12.0	8.8	106	57	.70	30
	11.8	11.0	129	69	.68	32
	12.8	11.0	140	45	.59	41
C.H.C. 74 kg	118.3	.42	50	276	.43	57
	111.0	.57	63	176	.43	57
	28.5	1.4	40	111	.21	79
	29.4	1.5	44	102	.31	69
	18.6	2.7	50	98	.35	65
	14.4	2.7	38	98	.33	67
	20.2	2.8	56	88	.39	61
	15.8	3.1	49	126	.29	71
	18.7	3.4	64	85	.42	58
	15.0	3.8	57	58	.42	58
	13.0	4.0	52	108	.29	71
	16.0	4.2	67	97	.36	64
	13.3	6.2	82	82	.41	59
	11.1	6.9	77	171	.28	72
	8.6	7.9	67	749	.35	65
	7.3	8.2	60	142	.35	65
	10.2	10.2	104	868	.43	57
	8.2	10.4	85	947	.53	47
	7.8	10.5	82	748	.44	56
	8.6	11.7	101	1,119	.45	55

* Clearance periods are arranged in order of increasing urine flow rates.

and subsequently corrected individual radiofluoride contents of each 4 ml sample of plasma and urine from subject CHC and presents them in their proper sequence. Particularly, it should be noted that plasma content of radiofluoride is relatively constant when compared to urine content of radiofluoride and that the latter appears to be correlated with urine flow rates. There is a notable concentration of urine radiofluoride when urine flow decreases below 1 ml/min. This circumstance tends to maintain a large urinary clearance of fluoride even when urine volume decreases.

Summary. One mg fluoride labeled with radiofluoride was ingested by each of 2 adult humans. Observations of renal clearance of fluoride, chloride and creatinine were made. Fluoride clearance always exceeded chloride clearance by many fold and increased with urine flow, but fluoride clearance was always less than creatinine clearance. Renal tubules in 2 individuals, reabsorbed respectively 51 and 63% of fluoride in the glomerular filtrate which indicates a net process of glomerular filtration with a variable amount of tubular reabsorption. Plasma contained 72% of whole blood radiofluoride and radiofluoride concentration of plasma exceeded that of parotid gland saliva. Measurement of uptake and release of the radioisotope by soft tissues and by the skeleton showed characteristic differences of radiofluoride retention in these tissues. Skeletal tissues retained the isotope but soft tissues lost nearly all their activity within 4 hours after ingestion.

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Antibacterial Substances in Placentas and Serums of Mothers and Newborn Infants. (25792)

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The protection of the human fetus against infection has been an intriguing subject for many years. Since the placenta interposes a continuous though narrow wall of tissue between the blood of the fetus and that of the mother, and since the chorionic cells of the placenta may be phagocytic(1,2), the simplest hypothesis would assign the role of a barrier to the placenta. The final destruction of invading organisms would be relegated to the antimicrobial substances of maternal and fetal bloods(3,4,5,6), and perhaps to some inadequacy of fetal tissues as a culture medium(7) for certain organisms. However, some reports (8,9,10,11) suggest that the placenta may contain antimicrobial substances distinct from those of the blood. The present report records concentrations of certain antibacterial substances in the blood of mothers and newborn infants and attempts to determine whether there are additional antibacterial factors in the placenta itself.

Materials and methods. Venous blood was obtained from mothers, and blood from newborn infants was collected from the placental portion of the severed umbilical cord immediately after birth. After the blood had clotted at room temperature serum was separated by centrifugation in the cold and stored at –20°C. Placenta were frozen at –20°C, allowed to thaw, comminuted in the cold for 3 minutes using a Waring blendor, and centrifuged at 2000 rpm for 1½ hours at 5°C. The sterile, dark red supernatant was stored at –20°C. Some placental preparations were treated with bentonite to remove lysozyme (12) or mixed with washed, heat killed bacteria to absorb specific antibodies. Bactericidal activity of the serums and placental materials were tested against *Escherichia coli, Shigella flexneri, Salmonella choleraesuis, Pseudomonas aeruginosa, Staphylococcus aureus, Streptococcus viridans* and a beta hemolytic *Streptococcus pyogenes*. Before testing, each organism was cultured for 24 hours in brain heart infusion broth (Difco). One-tenth ml of a 10^{-7} dilution of organisms suspended in 0.85% sterile NaCl solution was added to duplicate tubes containing either 1.0 ml cold serum, placental material or saline control. Mixtures were incubated for 30 minutes in a water bath at 37°C. Two ml molten nutrient agar (Baltimore Biological Labs.) was added to each tube, the contents mixed and allowed to solidify. *Pseudomonas* and *Salmonella* grew poorly below the surface of the agar. Mixtures containing either of these organisms were poured directly, therefore, onto the surface of nutrient agar plates. Colonies were counted after incubating tubes or plates for 24 hours at 37°C. By comparing colony counts of saline controls with counts of tubes containing placenta or serum, percentage of bacteria killed could be calculated. Hemolytic complement titers were expressed as the final dilution of serum or placental material required to lyse 50% of 1.5 x 10^8 optimally sensitized sheep erythrocytes in a reaction volume of 1.5 ml after 30 minutes of incubation at 37°C. Triethanolamine buf-

TABLE I. Bactericidal, Lysozyme, Properdin, and Complement Activities of Maternal Serums and Their Corresponding Cord Serum and Placenta.

Extract or serum	Bactericidal effect as % of *P. aeruginosa* killed in 30 min.	Lysozyme content (μg/ml)	Properdin (units/ml)	Hemolytic complement (C'H50/ml)
M*	98	7.0	8	233
C	96	8.4	4	146
Pl	78-95	13.4	1	13
M	100	5.8	16	<10
C	100	26.0	8	144
Pl	100	12.4	1	18
M	100	7.0	12	140
C	99	10.4	<2	68
Pl	100	9.4	2	17
M		5.6	8	231
C	100	6.4	4	100
Pl	75	8.4	1	20
M	90	4.4	8	48
C	90	6.4	<2	77
Pl	85	8.4	1	28
M	99	4.6	4	228
C	99	8.8	4	100
Pl	76	12.0	.5	<10
M	98	3.1	4	238
C	84	6.6	2	160
Pl	54	4.2	.5	22
M	98	4.6	8	100
C	100	6.6	2	—
Pl	99	14.4	2	26
M	94	4.2	<2	<10
C	100	8.0	3	114
Pl	50	9.6	2	25
M	100	4.0	4	112
C	75	7.4	<2	67
Pl	30	.5	<2	22
M	100	7.6	2	260
C	97	8.6	2	91
Pl	99	16.0	.5	26
M	100	5.3	8	196
C	100	9.0	<2	81
Pl	97	12.4	.5	17

* M, maternal serum; C, cord serum; Pl, placenta.

fered saline(13) containing Mg^{++} and Ca^{++} was used as the reagent diluent. Hemolysis was measured spectrophotometrically and results were plotted utilizing the probit transformation(14,15) to calculate the 50% hemolytic unit of complement (C'H50) per ml of sample. Lysozyme activity was determined by spectrophotometrically measuring the clearing of a suspension of *Micrococcus lysodeikticus*(16,17). Properdin assays were performed by the zymosan assay method of Pillemer, *et al.*(18). To detect properdin in placental extracts it was first necessary to precipitate properdin as euglobulin and resuspend it to one-fourth original volume in endpiece(13).

Results. Determinations of properdin, lysozyme, and bactericidal action against *Pseudomonas aeruginosa* were done on 20 maternal sera and the corresponding infant serums and placentas. In addition complement was determined in 12 of these cases. Representative results are shown in Table I. The maternal serums usually gave properdin values nearly equal to those of pooled normal serums and varying from 2 to 16 units. Infant serums from the umbilical cord had less properdin, and placental preparations had very small amounts, perhaps because of destruction of properdin by mechanical agitation. Lysozyme was found in all serums and placentas in concentrations resembling those in normal sera and ranging approximately from 5 to 25 μg/ml. In every case the serum of the newborn baby had a greater concentration of lysozyme than that of the mother. Complement titers in maternal sera were found to vary within the range for normal adults; complement titers were lower in cord sera than in maternal sera and greatly reduced in placental preparations, probably because of the agitation of the latter in the Waring blendor. Rarely, there was an unexplained deficiency of complement in maternal serum, but not in the corresponding placenta or neonatal serum, a finding which could not be correlated with the patient's blood group or obstetrical history. All serums and placental preparations demonstrated bactericidal activity. This activity against *Pseudomonas aeruginosa* could not be correlated closely with levels of properdin, complement or of lysozyme (Table I).

When tested against 7 different organisms, each placental preparation showed a distinct spectrum of bactericidal activity (Table II), particularly against *Shigella flexneri*, *Escherichia coli* and *Pseudomonas aeruginosa*, but no detectable activity against *Salmonella choleraesuis*, *Streptococcus pyogenes* or *Streptococcus viridans*. Only one placenta and cord serum showed activity against *Staphylococcus*

TABLE II. Antibacterial Spectra of Placentas and Serums.

Serum	*Staph. aureus*	*Strep. viridans*	*Strep. pyogenes*	*Esch. coli*	*Shigella flexneri*	*Salmonella choleraesuis*	*Pseudomonas aeruginosa*
M*	0	0	0	+	+	0	+
C	0	0	0	+	+	0	+
Pl	0	0	0	+	+	0	+
M	—	0	—	—	0	—	—
C	0	0	0	+	+	0	+
Pl	0	0	0	+	+	0	+
M	0	0	0	+	+	0	+
C	0	0	0	+	+	—	+
Pl	0	0	0	+	+	+	+
M	0	0	0	+	+	0	+
C	0	0	0	+	+	0	+
Pl	0	0	0	+	+	0	+
M	0	0	0	+	+	0	+
C	0	0	0	+	+	0	+
Pl	0	0	0	+	+	0	+
M	0	0	0	+	—	0	+
C	+	0	0	+	0	0	+
Pl	+	0	0	+	0	0	+
M	0	0	0	0	0	0	0
C	0	0	—	—	+	0	—
Pl	0	0	0	+	0	0	0
M	0	0	0	+	0	—	—
C	0	0	0	+	0	0	+
Pl	0	0	0	+	0	0	0

* M, maternal serum; C, cord serum; Pl, placenta.

+, more than 50% of organisms killed in 30 min. at 37°C.; 0, few or no organisms killed; —, not tested.

aureus. Several placentas were tested against *Candida albicans* and found completely inactive. The antibacterial spectrum of each placental preparation was almost identical with that of the neonatal and maternal serums from the same patient.

Heating at 56°C for 30 minutes completely inactivated the antibacterial substances of the placenta. *Pseudomonas aeruginosa* and *Escherichia coli* survived as well in heated placental material as in 0.85% NaCl solution.

Absorption with killed bacteria also resulted in loss of bactericidal activity, but usually against the absorbing organism only. A placental preparation absorbed with *Escherichia coli* killed *Pseudomonas* but not *Escherichia coli*. A preparation absorbed with *Shigella* was ineffective against *Shigella* but remained strongly bactericidal for *Escherichia coli* and *Pseudomonas*. When the antibacterial effect of a placental preparation had been lost by specific absorption, it could be restored by addition of heat-inactivated placenta (Table III).

Variations in pH, from 6.01 to 7.88 had little effect on bactericidal activity of placental extracts. Treatment with bentonite reduced concentration of lysozyme to less than 0.5 μg/ml without affecting bactericidal activity. Likewise, wide variations in concentration of properdin and complement had little effect (Table I).

Discussion. The human placenta, familiar as a source of gamma globulin, has been shown to contain lysozyme, properdin, and complement. The presence of these substances was expected, as they are normal constituents of blood, and the placenta is a very vascular organ. Since levels of properdin and complement were higher in maternal than neonatal serum, it may be conjectured that both factors are passively transferred through the placenta to the fetus and not produced or concentrated by the placenta.

On the other hand, lysozyme was present in greater concentration in the blood of the infant than in that of the mother. This unexpected finding suggests that lysozyme is either

TABLE III. Bactericidal Activity of Placental Preparations against *Escherichia coli.*

	Dilution of placenta				
	1:4	1:8	1:16	1:32	1:64
Placenta	+	+	+	+	+
" absorbed with *E. coli*	0	0	0	0	0
" heated 56° 30 min.	0	0	0	0	0
Heat-inactivated placenta + placenta absorbed with *E. coli*	+	+	+	+	0

+, definite bactericidal effect, more than 80% of organisms killed; 0, no definite effect, more than 80% survived.

selectively transmitted across the placenta or produced in the fetal organism or its membranes. It is of interest that in certain instances, antibodies also have been found in greater concentration in cord serum than in maternal serum(19).

No evidence of a distinct antibacterial substance in placental tissue itself was found. In spectrum, or selectivity of action against various species, bactericidal effects of each placental preparation were similar to those of the maternal and neonatal serums from the same patient. Bactericidal activity was relatively independent of concentrations of properdin, lysozyme, and complement, but depended on presence of a heat-stable specific factor, which may be regarded as antibody, and a heat-labile non-specific factor, probably bactericidal complement. In all these respects antibacterial activity of the placenta resembles that of normal mammalian serums (20). Thus, this activity of the placenta appears to be derived from the blood, which forms a large fraction of the bulk of the organ as it is delivered.

Summary. 1. Serum was obtained from umbilical cords of healthy newborn babies and from their mothers just after delivery. Liquid preparations of placentas were produced by freezing, thawing, comminution and centrifugation. When tested against 7 species of bacteria, sera and placenta showed bactericidal activity, particularly against Gram negative forms. Placenta and cord serum were active against the same organisms as maternal serum from same patient. 2. Maternal and cord sera and placentas contained lysozyme, complement, and properdin. Placenta and cord serum showed a higher concentration of lysozyme than maternal serum. Placental preparations contained less complement and properdin than serums. 3. Heating at 56°C for 30 minutes caused complete loss of bactericidal activity in placental preparations. Absorption with killed bacteria usually caused loss of bactericidal activity, generally against the absorbing organism only, and this specific bactericidal activity could be restored by addition of heated placenta. 4. Placenta contains antibacterial substances of blood but seems to have little or no detectable bactericidal substance of its own. Apparently it protects the fetus from bacterial infection largely by transmitting protective substances from mother and mechanically impeding access of bacteria to the fetus.

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Cushing's Syndrome V. Chemical Inhibition of 11β-Hydroxylating Enzyme Systems in Hyperplastic and Neoplastic Adrenal Glands.[*] (25793)

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The clinical distinction between adrenal hyperplasia and adrenal tumor as the cause for Cushing's syndrome has been based in part upon tests which inhibit ACTH secretion (1), and also upon response of the adrenal glands to stimulation with exogenous ACTH (2-4). In general, adrenal tumors are neither suppressed by exogenous steroids nor stimulated with ACTH, whereas hyperplastic glands usually hyper-respond to stimulation with ACTH and are suppressed by administration of exogenous corticosteroids. However, these tests do not permit absolute distinction between the two pathological states (5-7). Su 4885, (2-methyl-1, 2-bis-(3-pyridyl)-1-propanone),[‡] when administered in proper dosage to the human subject, selectively causes inhibition of 11β-hydroxylation within the adrenal cortex. This substance has been extensively studied in the normal individual and in patients with adrenal and pituitary dysfunction(7,8). Differences in response to Su 4885 have been noted to occur between patients with adrenal tumors and those with bilateral hyperplasia(8). The purpose of this investigation has been to define the action of Su 4885 upon the biosynthetic mechanisms within the adrenal gland in Cushing's syndrome when due to either bilateral adrenal hyperplasia or neoplasia.

Methods and materials. In all, 7 patients with documented Cushing's syndrome were studied. Two patients were shown to have adrenal carcinoma and 5 to have bilateral adrenal hyperplasia. The urinary ketogenic steroids were determined by a modification of the Norymberski technic(9). The methods for extraction, separation and identification of the various corticosteroid metabolites have been reported(10). In brief, urinary steroids after extraction were separated by chromatography on Bush$_1$ and Bush$_5$ systems. Eluted steroid fractions were quantitated by a modified Porter-Silber reaction. The tetrahydro S[§] metabolite was identified by melting point determinations and infrared spectra, together with similarities in Rf values to a standard on several different chromatographic systems. Twenty-four-hour urine collections were obtained for several days before administration of the Su 4885 compound and for several days thereafter. 750 mg of Su 4885 were administered every 4 hours for 6 doses except in the case of St. who received a total of 200 mg in this period of time.

Results. Adrenal hyperplasia. Administration of Su 4885 over a one day period to 5 patients with bilateral adrenal hyperplasia and Cushing's syndrome resulted in a marked increase in urinary ketogenic steroids (Table I). In 4 of these 5 patients, maximal rise occurred the day following administration of Su 4885. In the fifth patient, Gu., maximal increase was observed on day of administration of the compound.

The results of identification and quantitation of the various hydrocortisone and com-

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[*] Aided by grant, N.I.H., U.S.P.H.S.

[†] Trainee, N.I.H., U.S.P.H.S.

[‡] Kindly supplied by Dr. Neil Sullivan, Ciba Pharmaceutical Co.

[§] $3\alpha, 17\alpha,21$-triol, pregnan-20-one.

TABLE I. Effect of Su 4885 on Urinary Ketogenic Steroid Excretion in Patients with Cushing's Syndrome.

	Patient	Sex	Age	Ketogenic steroids (mg/24 hr) Day 1	Day 2*	Day 3	Day 4
Adrenal hyperplasia	Ma.	♀	61	34	29	66	
	Ho.	♀	40	31	44	101	
	Co.	♂	51	28	39	65	
	Gu.†	♀	52	46	61	41	
	Gs.	♀	38	22	49	85	
" carcinoma	Fl.	♂	33	40	39	25	28
	St.	♂	1½	12	5	7	12

* Su 4885 administered in divided doses on Day 2.
† Unilateral adrenalectomy performed 6 yr previously.

TABLE II. Effect of Su 4885 on Individual Urinary C-21 Steroids in Patients with Cushing's Syndrome.

		Tetrahydro F, tetrahydro E & allotetrahydro F	Tetrahydro S	Dihydro S	Total
Adrenal hyperplasia	Before Su 4885	27.2	1.1	0	28.3
(patient Ho.)*	After "	2.6	27.5	12.7	42.8
Adrenal carcinoma	Before "	31.5	1.6	0	33.1
(patient Fl.)	After "	5.8	12.3	0	18.1

* This study upon patient Ho. was performed prior to that reported in Table I.

pound S metabolites in a representative patient are shown in Table II. Tetrahydro F[‖], tetrahydro E[¶] and allotetrahydro F[**] fell from a control level of 27.2 mg/day to 2.6 mg/day, whereas values for urinary tetrahydro S rose from 1.1 to 27.5 mg/day. These changes reflect inhibition by Su 4885 of 11β hydroxylation within the hyperplastic adrenal gland. A compound tentatively identified as dihydro S[‡‡], on the basis of running rates similar to the authentic compound in several chromatographic systems, appeared in the urine as a result of Su 4885 administration to patient Ho.

Adrenal carcinoma. Administration of Su 4885 to 2 patients with adrenal carcinoma resulted in an actual decrease in excretion of urinary ketogenic steroids (Table I). Analysis of specific urinary steroids in one patient showed a decrease in tetrahydro F, tetrahydro E and allotetrahydro F levels from 31.5 mg/day to 5.8 mg/day following administration of the compound, whereas the level of tetrahydro S rose from 1.6 to 12.3 mg/day. However, total amount of identifiable steroid metabolites in the urine fell from 33.1 to 18.1 mg/day (Table II).

Discussion. In a patient with Cushing's syndrome due to bilateral adrenal hyperplasia, as in the normal individual, administration of adequate amounts of Su 4885 results in a definite increase in urinary ketogenic steroids. This increase is caused by a marked rise in tetrahydro S excretion and not by an increased excretion of hydrocortisone metabolites. In fact, the latter group of steroids decreases after Su 4885 administration. Su 4885 apparently causes a decrease in amount of circulating hydrocortisone, thereby diminishing the usual inhibiting influence of hydrocortisone upon the pituitary gland. The pituitary consequently secretes excessive amounts of ACTH, which in turn stimulate the adrenal cortex. However, as the 11β-hydroxylating enzyme systems are inhibited, the adrenal glands secrete large amounts of compound S rather than hydrocortisone. The metabolites of compound S are identified as ketogenic steroids in the urine.

Following administration of Su 4885, the metabolites of the hydrocortisone in the urine of patients with adrenal carcinoma decrease

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[‖] 3α,11β,17α, 21-tetrol, pregnan-20-one.

[¶] 3α,17α,21-triol, pregnane-11, 20-dione.

[**] 3α,11β,17α,21-tetrol, allopregnan-20-one

[‡‡] 17α,21-diol, pregnane-3, 20-dione.

in a manner similar to the response of patients with adrenal hyperplasia and normal individuals. Therefore, it seems clear that Su 4885 inhibits 11β-hydroxylation in adrenal carcinoma as well as in non-neoplastic glands. The difference in response as regards total urinary ketogenic steroids apparently resides in the failure of the neoplastic glands to respond to stimulation with the endogenous ACTH released after Su 4885 administration. Patients with carcinoma of the adrenal usually do not show rises in plasma or urinary steroid levels when ACTH is administered, nor do their urinary steroids diminish following suppression of ACTH with exogenous steroids.

The response to Su 4885 may prove useful as a clinical test for differentiating adrenal carcinoma from hyperplasia(8) but more data must be amassed before generalizations can be made. Analogs of this compound may also assume a role in the chemotherapy of adrenal carcinoma.

Summary. Administration of Su 4885 resulted in inhibition of 11β-hydroxylation within the adrenal cortex in 7 patients with Cushing's syndrome. In 5 patients with adrenal hyperplasia, this resulted in a rise in values of urinary ketogenic steroids whereas in the 2 patients who had adrenal carcinoma no such rise was observed. This difference in the 2 groups of patients is ascribed to the state of adrenal responsiveness to endogenous ACTH.

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In vitro Passive Transfer of Atopic Hypersensitivity. (25794)

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During experiments on allergic histamine release from leukocytes of atopic subjects(1-3), it was shown that histamine release varies in relation to antigen concentration(1). To study the effect of antibody concentration, attempts were made *in vitro* passively to sensitize leukocytes from non-allergic individuals. That this might be done successfully seemed reasonable in view of the fact that normal skin is regularly sensitized by allergic sensitizing antibody. This is the basis of the Prausnitz-Küstner reaction. Also, Humphrey and Jacques(4) demonstrated that normal rabbit platelets will release histamine in presence of homologous rabbit antibody and antigen.

Methods. Heparinized whole blood was obtained from normal skin test negative individuals. Plasma containing sensitizing antibody was procured from subjects highly allergic to ragweed pollen (3-4 plus reaction to skin test with 1/40 ml ragweed extract containing 100 protein N units per ml). The capacity of ragweed antigen to produce histamine release in the whole blood of the donor subjects is shown in Table I. In some experiments donor plasma was diluted with 0.15 M NaCl prior to mixing with normal whole blood. In other experiments, plasma was col-

TABLE I. Histamine Release in Blood of Ragweed Sensitive Subjects Whose Plasma Was Used for Passive Transfer.

	Plasma histamine conc., μg/l after addition to whole blood of				
		Ragweed (final conc., μg protein N/l)			
Subjects	Saline	2	10	20	50
Gor.	0			69	
	0			70	
App.	0	16			
	4			119	
Ben.	6	36	52		25*
Ten.	5		48		
Dow.	15				175
Kas.	7			93	12*

* Less than maximum histamine release occurs in high antigen excess(1).

lected from the allergic subjects' blood after addition of various amounts of ragweed antigen to the whole blood, *i.e.*, after the histamine release had taken place. The pairs of individuals (donor and acceptor) were matched according to major blood groups (but for one exception, see Table II). To 36 ml of heparinized normal whole blood was added 4 ml of sensitive plasma, plasma dilution, or plasma from ragweed-treated sensitive whole blood. The mixtures were incubated at 37°C for 2 hours with occasional mixing. At this point, saline was added to a control tube and ragweed extract to the other, to give a final concentration of 50 μg protein N per liter. After 30 minutes incubation at 37°C the tubes were centrifuged and plasma collected and analyzed for histamine as previously described(1).

Results. In 10 attempts with 6 sensitive plasmas and 7 normal bloods, only 3 pairs of individuals provided systems which demonstrated passive sensitization as determined by histamine release. As a check 2 pairs were retested and reproducibility was established. Table II shows this information and also demonstrates that one sensitive plasma may not sensitize every acceptor exposed to it (*e.g. Gor* plus Chr +; *Gor* plus Wil –). It further shows that cells of one positive acceptor (Gor plus *Chr* +) may not be sensitized by another sensitive plasma (Dow plus *Chr* –). The explanation of this phenomenon is not known.

Nevertheless, in addition to these interesting findings, several other points are worthy of mention. First is the diminution of histamine release from cells sensitized with plasma dilutions (Table II). This is consistent with the view that sensitizing antibody concentration is one of the limiting factors determining amount of histamine released at constant cell and antigen concentration.

TABLE II. Histamine Release from Passively Sensitized Normal Leukocytes.

System		Histamine released,* μg/l of plasma from normal whole blood plus		
Ragweed sensitive plasma (donor)	Normal cell source (acceptor)	Sensitive plasma (untreated)	Sensitive plasma dilution	Plasma from treated sensitive whole blood¶
Gor. (A+)**	Chr. (A–)	68	0 (1:10)	15§
	" "	50		22§
	Wil. (O+)	0		
App. (A+)	Lar. (A–)	53	23 (1: 2)	39†
	" "	54		15§
Ben. (O+)	Wat. (O+)	19	5 (1: 5)	9,† 8,‡ 0‖
Ten. (A+)	Cra. (A+)	2		
Dow. (A–)	Chr. (A–)	0		
	Whe. (A+)	0		
Kas. (A+)	Whi. (A+)	0		

* Ragweed extract added to final mixtures of normal whole blood-sensitive plasmas to give a concentration of 50 μg protein N/l.

† Prior treatment of sensitive whole blood with 2 μg ragweed protein N/l.
‡ *Idem* 10
§ " 20
‖ " 50

¶ Saline controls ranged between 8-15 μg histamine/l. These are normal values for control plasma.

** Blood group notation; A+ is group A, Rh positive, etc.

Second is the finding that plasma obtained from whole blood previously treated with ragweed antigen confers a diminished capacity upon normal cells to release histamine. The larger the quantity of ragweed used for prior treatment, the less histamine release occurred on subsequent sensitization of normal cells with such plasmas (Table II). This observation strongly suggests that in the initial treatment of sensitive whole blood with ragweed antigen that sensitizing antibody is actually used up in some fashion and hence there is less remaining for subsequent sensitization of the normal cells. Thus, less histamine release occurs on later addition of ragweed to treated plasma-normal cell mixtures. Diminution in transferable sensitizing antibody activity could occur in at least 3 different ways: 1) humoral antibody complexing with antigen, thus preventing reaction of cellular antibody and antigen and subsequent histamine release; 2) binding of antibody to cells during the course of histamine release reaction; 3) formation of a product in the release reaction which inhibits histamine release in a fresh reaction system.

Had the interaction of sensitizing antibody, antigen and cells in the sensitive blood caused generation of any stable anaphylatoxin-like substance, then addition of plasma from such a system to normal blood might be expected to cause a release of histamine without further addition of ragweed antigen. The last column of Table II shows that such plasmas added to normal blood without antigen (saline control) do not bring about a significant release of histamine. It appears, therefore, that this type of allergic reaction is not accompanied by anaphylatoxin generation.

Summary. *In vitro* sensitization of non-allergic human leukocytes by plasma from ragweed sensitive subjects was carried out. In 10 attempts with 6 sensitive plasmas and 7 normal bloods, only 3 pairs of individuals provided systems which demonstrated passive sensitization as determined by histamine release in presence of ragweed antigen. This finding is not related to major blood group incompatibility, and is unexplained. Diluted sensitive plasma brings about less histamine release. Plasma obtained from sensitive whole blood previously treated with ragweed antigen confers a diminished capacity upon normal cells to release histamine. There was no evidence that such plasmas possessed anaphylatoxin-like activity.

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Method of Concentration of Viral Diagnostic Reagents Using Hydrophilic Agents. (25795)

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A number of methods other than procedures primarily dependent upon high speed centrifugation and lyophilization have been employed for concentration of viral and rickettsial suspensions. Several chemical methods have been described(1,2,3,4,5). Kohn(6) outlined a procedure for concentration of urine using polyvinylpyrrolidone (PVP)* and polyethylene glycol (Carbowax 20-M)† as hydrophilic agents. It occurred to us that with certain modifications Kohn's general method would be of possible value in the production of viral diagnostic reagents.

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* K and K Laboratories, Jamaica, N. Y.

† Carbide and Carbon Chemicals Co., Union Carbide and Carbon Corp., N. Y.

Use of trade names does not imply endorsement by Public Health Service.

Materials and methods. Antigens. The following complement-fixing (CF) antigens were prepared for concentration experiments: mumps viral and mumps soluble(7), influenza A/PR8/34, influenza A2/Jap/305/57, influenza B/GL/1739/54, and influenza B/Maryland/1/59(8), lymphocytic choriomeningitis (LCM)(9), and poliovirus types 1 (Mahoney strain), 2 (Statler strain), and 3 (McMullen strain)(10). Before concentration the poliovirus CF antigens were treated with fluorocarbon (Genetron 113‡ was substituted for the Freon 112-*n*-heptane mixture described by Hamparian *et al.*(11)) to remove possible anticomplementary activity. The other antigens were concentrated with no prior treatment because they were not found to be anticomplementary. These antigens were selected because they represented 3 sources of host material: egg (mumps and influenza antigens), tissue culture (poliovirus antigens), and animal tissue (LCM antigens). *Concentration procedure.* To maintain optimal aseptic technic and facility of manipulation of large volumes, the following modification of Kohn's technic was utilized. An appropriate length (depending on volume of antigen used) of cellulose dialyzer tubing was fastened to a small funnel by cotton string and knots tied in the distal end of the tubing. The unit was sterilized in the autoclave. Antigen was added aseptically to the tubing, the funnel was removed, and the upper portion of the tubing was tied securely with cotton string. The tubing was then placed in a suitable container (battery jar, cylinder, beaker, etc.) and the top of the tubing was secured with adhesive tape to the top of the wall of the container in such manner that the fluid dialyzing out of the antigen within the tube would not ultimately reach the top portion of the membrane. The lower portion of the tubing was coiled so that it lay flat in bottom of the container. Carbowax (polyethylene glycol compound 20-M), in a ratio of 1 g to 9 ml of the antigen within the dialyzer tubing, was sprinkled over the entire outer surface of tubing. The assembly was kept at +4 to +8° usually overnight and in some cases for a longer period. Concentration is effected in shorter time periods when larger amounts of the agent are added, especially if the antigen is distributed in more than one dialyzer tube. Ratio of hydrophilic agent to volume of antigen to be concentrated is not critical. It has been possible to achieve a 10-fold volume concentration of several antigens within a 24-hour period. The product was finally removed from the dialyzer tubes in an aseptic manner using a bulbous beveled point (Horsley) needle and suitable syringe. *Serological methods.* Hemagglutination (HA) tests were performed in tubes using chicken erythrocytes and the ensuing cell pattern was read after a period of 1 hour at room temperature. The CF test employed in this study was that described by Sigel *et al.*(12) for all antigens other than poliovirus. The CF test procedure outlined by Schmidt and Lennette(13) was followed for testing poliovirus CF antigens. Titers were recorded as the reciprocal of the highest dilution giving the endpoint of titration.

Results. An increase in serologic activity was effected by concentration with PVP or Carbowax using both CF and HA tests. This concentration with increased serologic activity was demonstrated using all 3 categories of host material for antigen production. Volume concentration was effected from 2.8 to 22.4-fold of the original material. When the increases in HA and CF titers were compared for different lots of each type of antigen concentrated, there seemed to be no consistent relationship with the exception of 2 lots of influenza B/GL/1739 antigens. Here, 4-fold increases in titer were observed in both lots of antigen using 2 serologic technics with a 10-fold decrease in volume. In the case of the 2 lots of mumps soluble antigen, the original preparations showed negative HA tests in the tubes of undiluted antigen but elicited an HA pattern in high titers in both concentrates. The results are summarized in Table I.

Discussion. Methods describing concentra-

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‡ Genetron trifluorotrichloroethane Purple label 113. Allied Chemical and Dye Corp., N. Y.

TABLE I. CF and HA Antigen Titers before and after Concentration.

Antigen	Vol in ml,* orig./conc.	Fold conc.	HA titers		CF titers	
			Conc./orig.†	Fold increase	Conc./orig.†	Fold increase
Mumps						
Viral: Exp. 1	590/210	2.8	64/32	2	8/2	4
2	1,060/101	10.5	256/32	8	8/<2	>4
3	730/73	10.0	512/64	8	32/2	16
4	640/64	”	1,024/64	16	16/4	4
Soluble: Exp. 1	386/37	10.4	32/undil.	>32	8/4	2
2	125/11	11.4	256/ ”	>256	32/8	4
Influenza						
A/PR8/34: Exp. 1	600/80	7.5	512/128	4	16/2	8
2	365/20	18.2	16,384/512	32	64/4	16
A2/Jap/305/57:						
Exp. 1	515/23	22.4	4,096/512	8	128/8	”
2	570/57	10.0	2,048/512	4	”	”
B/GL/1739/54: Exp. 1	128/12	10.7	512/128	4	16/4	4
2	82/8	10.2	1,024/256	4	32/8	4
B/Maryland/59:						
Exp. 1	500/56	8.9	” / ”	4	4/2	2
2	710/70	10.1	4,096/512	8	16/4	4
3	670/63	10.6	” / ”	8	”	4
LCM						
Exp. 1	130/13	10.0			256/16	16
2	150/15	”			512/32	”
Poliovirus						
Type 1: Exp. 1	300/50	6.0			48/4	12
2: 1	”	6.0			24/2	”
2	470/80	5.9			10/2	5
3	600/50	12.0			”	5
4	750/80	9.4			12/4	3
3: 1	300/50	6.0			24/4	6
2	970/120	8.1			6/2	3
3	700/50	14.0			10/4	2.5
4	825/60	13.7			6/3	2

* Numerator = Original vol. Denominator = Concentrated vol.
† Numerator = Titer after concentration. Denominator = Titer before concentration.

tion of viruses and viral antigens employing high speed centrifugation which have been conveniently utilized where volumes are small are usually impractical when large volume production methods are used. Methanol precipitation methods, although generally successful for concentration of mumps viral, influenza, and poliovirus CF antigens in this laboratory, have disadvantages of 1) excessive manipulation in the process, 2) observation of thermal restrictions, and 3) loss in final yield of serologic activity in some cases. Furthermore, optimal methanol concentration and pH of the eluting menstruum have to be ascertained. The method described here requires no special equipment. It can be easily applied to antigens eliciting various ranges of titers. Presumably, no chemical contamination takes place other than that which is present in the hydrophilic agent used. However, it should be realized that a concentration of all non-dialyzable components in the antigen also takes place. Indeed, the appearance of cloudy material was noted in some of the egg antigen concentrates. This was absent in the original fluids. However, none of the antigens tested with the CF technic were found to be anti-complementary after concentration. It has been noted during these experiments that another method of virus concentration has been described recently(14) using the principle of partition of viral agents in dextran-methylcellulose-water and dextran-Carbowax 6000-water systems.

Summary. A simple and economical method is described for concentration of viral

diagnostic antigens. Evidence of serologic increase in titer has been presented with influenza, mumps, lymphocytic choriomeningitis, and poliovirus antigens.

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Carbohydrate Constituents of Human Red Cell Stroma.*† (25796)

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Information concerning the carbohydrates in red cell stroma is scanty. Most of the work in this field has been done on specific compounds prepared from stroma. This paper is concerned with analysis of monosaccharides, hexosamines and sialic acid in human red blood cell stroma. In addition, studies were conducted to determine the effect of storage of blood in the cold on carbohydrate constituents.

Methods. Bloods obtained from 4 healthy young men were collected in acid-citrate-dextrose and aliquots were stored in the cold (4°C) under sterile conditions in stoppered bottles. Zero day samples and bloods stored for 15, 30 and 45 days were treated by the short-wash procedure for preparing stroma from dog erythrocytes described by Tishkoff *et al.*(1). This material was lyophilized and kept under vacuum over P_2O_5. Total hexose content was determined by the anthrone method(2). It was found essential to age the anthrone reagent for at least 24 hours to obtain constant results. Dried stroma (10 mg) was hydrolyzed in 3 ml 2N H_2SO_4 in a tube stoppered with a screw-cap lined with rubber. The tube was slowly rotated for one-half hour in a water bath maintained at 96°. Insoluble material was removed by centrifugation and clear supernatant was used for analysis. Galactose in 2N H_2SO_4 was used as a standard and values were expressed as galactose. To separate the lipid-carbohydrate complexes, dried stroma (25 mg) was extracted for one hour with occasional shaking with 10 ml of a chloroform-methanol mixture (2:1)(3) at room temperature. The stroma residue was re-extracted 3 times with one ml portions of the chloroform-methanol mixture; extracts were pooled and evaporated to dryness in a stream of nitrogen. The residual stroma and the extracted material were hydrolysed with 2N H_2SO_4 and analysed for hexoses. To obtain clear hydrolysates, it was necessary to remove the solution carefully so as to avoid the insoluble material which floated on the surface. Hexosamine was determined by the Boas method(4). Stroma (20 mg) was hydrolyzed for 5 hours in 2 ml 2N H_2SO_4 in a screw-cap tube rotated in a water bath at 96°. The hydrolysate was diluted to 8 ml and cen-

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† The author acknowledges assistance of Harry H. Hallatt.

TABLE I. Total Hexose Concentration of Stroma.

Blood	Whole stroma 1	2	3	4	Lipoidal extract of stroma 1	2	3	4
Days of storage	g/100 g							
0	2.0	1.8	1.9	1.9	.40 (20)	.43 (24)	.45 (24)	.39 (21)
15	1.9	1.9	1.9	1.8	.41 (22)	.39 (21)	.49 (26)	.43 (24)
30	2.0	1.9	2.1	1.9	.44 (22)	.43 (23)	.47 (22)	.35 (18)
45	1.9	1.7	2.0	1.8	.36 (19)	.36 (21)	.44 (22)	.40 (22)

Figures in parentheses represent % of total hexose.

trifuged. Two ml of this solution was found to be satisfactory for application on the Dowex 50 column. Sialic acid was determined by the thiobarbituric acid assay of Warren(5). Stroma (10 mg) in 2 ml 0.1N H_2SO_4 was heated for one hour in a screw-cap tube while rotating slowly in a water bath at 80°(6). Acetylneuraminic acid, kindly supplied by Dr. Leonard Warren, was used as a standard. The monosaccharides in the stroma hydrolysate were determined by paper chromatography. The first eluate passing through Dowex 50, which is discarded in the Boas method(4), was used. Eluates from 4 runs were combined and concentrated to 5 ml in a desiccator containing NaOH pellets. This concentrated solution was transferred to a 5 ml round bottom beaker and evaporated to dryness in a desiccator; the material was dissolved in 20 μl water and 5 and 10 μl portions applied to Whatman #2 paper. The sugars were chromatographed with an n-butanol-pyridine-water (45:25:40) mixture(7) for 36 hours and developed by spraying with aniline phthalate(8). Reference sugars were run at the same time.

Results. Values for the total hexoses of stroma prepared from freshly drawn blood and from blood stored in the cold for 15, 30 and 45 days are presented in Table I. Hexose concentration is about 2% in 4 different bloods and values are not affected by storage. Chloroform-methanol extracts of these stromas contained about 22% of total hexoses (Table I). Lipid-carbohydrate complexes, which are extracted from stroma by fat solvents, have been found to be rich in hexoses (9,10).

TABLE II. Hexosamine Concentration of Stroma Expressed as Glucosamine-HCl.

Days of storage	Blood 1	2	3	4
	g/100 g			
0	1.2	1.1	1.2	1.2
15	1.2	1.3	”	”
30	1.3	1.2	”	
45		1.3		1.3

Values for hexosamine-HCl concentrations vary between 1.1 and 1.3 g % in the stroma of fresh and stored blood (Table II). It has not been possible to find data in the literature concerning concentration of hexosamine in stroma. McCrae(11) has demonstrated that a mucoprotein isolated from human red cell stroma contains appreciable amounts of hexosamine.

TABLE III. Sialic Acid Concentration of Stroma Expressed as Acetylneuraminic Acid.

Days of storage	Blood 1	2	3	4
	g/100 g			
0	1.1	1.2	1.2	1.2
15	”	1.3	”	1.3
30	”	”	”	”
45	1.3	”	1.4	1.2

Table III shows that the sialic acid concentration is about 1% and does not change appreciably during 45 days of storage. The absorption spectrum of the assay chromophore of a number of stroma hydrolysates showed only one maximum at 549 mμ, which indicates that no interfering substances were present. In a few samples sialic acid was determined by the anion exchange resin method of Svennerholm(6) and values were close to those obtained by the Warren method. Klenk and Lempfried(12) demonstrated the presence of about 1% neuraminic acid in stroma of human erythrocytes which is in close agreement with values obtained in the present experiments.

The constancy of concentrations of hexoses, hexosamine and sialic acid of stroma obtained from red cells stored over a 45-day period indicates that these sugars are not involved in the metabolic processes of erythrocytes.

Qualitative analysis of the acid hydrolysates of stroma by paper chromatography showed the presence of large amounts of galactose, smaller amounts of glucose and mannose and a trace of fucose. Excellent resolution without trailing was observed for each of the sugars. A number of investigators(9,10) found galactose and glucose present in stroma. In addition, McCrae(11) found fucose present in his mucoprotein preparation. This appears to be the first study in which mannose is reported to be present in stroma.

Summary. Human red cell stroma has been found to contain 2% hexoses, 1.2% hexosamine and 1.2% sialic acid. These stroma values remain constant during storage of whole blood in the cold for 45 days. Monosaccharides found in stroma were galactose, glucose, mannose and fucose.

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Serumless Medium for Cultivation of Cells of Normal and Malignant Origin.* (25797)

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Development of serum-free media for growth of cells in culture generally has culminated in a medium containing a protein fraction from serum or one suitable for "adapted" cell strains. Waymouth grew strain "L" mouse fibroblasts in media in which Armour fraction V and peptone were substituted for serum(1). Fisher *et al.* successfully grew colonies of human cells in media containing serum albumin and fetuin(2). Lieberman and Ove demonstrated the growth promotive properties of insulin, serum "sticking" factor and catalase in media containing serum albumin(3). Certain lines of strain "L" mouse fibroblasts have been cultivated in completely defined media(4,5,6). Recently, monkey kidney(7), Chang liver cells(8) and mouse lung(9) have been adapted to similar media sometimes supplemented with peptone. The present report concerns growth of a variety of cell cultures in media devoid of serum proteins and without a preliminary period of adaptation.

Methods. One ml of whole medium containing 10,000-15,000 Walker carcinosarcoma 256, KB, human heart, sarcoma 180 cells or 100,000 primary green monkey kidney cells was pipetted into 16 x 125 mm tubes placed in a slanted position in racks. The cultures were established (plated) by incubation for 24 hours at 37°C in an atmosphere of 8%

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We acknowledge excellent technical assistance by Joan Molowski.

TABLE I. Serumless Medium for Growth of Cell Cultures.

Constituent	Quantity	Constituent	Quantity	Constituent	Quantity
Amino acids	(mM/l)	Vitamins	(μg/ml)	Minerals	(g/l)
L-alanine	.1	Ascorbic acid	.5	NaCl	6.46
-arginine • HCl	.2	Biotin	.2	$NaHCO_3$	2.20
-asparagine • H_2O	.3	B_{12}	.00075	Na_2HPO_4	.14
-aspartic acid	.1	Calcium pantothenate	.2	KCl	.40
-cysteine	.2	Choline • HCl	5.0	$CaCl_2$	.20
-glutamic acid	.1	Folic acid	2.0	$MgSO_4 \cdot 7H_2O$	.20
-glutamine	1.5	Glutathione	.5	Glass distilled water	to 1000 ml
glycine	.1	Inositol	9.0		
-histidine • H_2O • HCl	.1	Nicotinamide	.5	Special additives	(mM/l)
hydroxy-L-proline	.1	Pyridoxine • HCl	.5	Pyruvate	1.0
-isoleucine	.3	Pyridoxal • HCl	.5	Mucate	.1
-leucine	.3	Riboflavin	.2		
-lysine • HCl	.2	Antibiotics			(μg/ml)
-methionine	.1	Penicillin G, sodium	50	Salmine sulfate	5.0
-phenylalanine	.1	Streptomycin sulfate	50	Insulin*	1.0
-proline	.1	Indicator		Methyl oleate†	15.0
-serine	.25	Phenol red	2.5	Folinic acid	.1
-threonine	.1				(mg/ml)
-tryptophan	0.03	Carbohydrates	(g/l)	Lactalysate‡	2.0
-tyrosine	.1	Glucose	3.0		
-valine	.1				

All constituents except salmine, insulin and methyl oleate are dissolved together in balanced salt solution (Earle's solution). Salmine sulfate, methyl oleate and insulin are added separately.

* Crystalline zinc insulinate, 23.6 U.S.P. units/ml.

† Tween 80 (15.0 μg/ml) and ETOH (600 μg/ml) used to dispense oleate.

‡ EDAMIN DR, (pancreatic digest of lactalbumin), Sheffield Chemical, Norwich, N. Y.

carbon dioxide-92% air. Medium was removed and tubes were rinsed with Earle's solution. The cells then were overlaid with 1 ml of test medium containing graded amounts of the test compounds. Incubation was continued for 3-4 days until cells attained maximal growth in presence of optimal amounts of test compounds. The medium was not replenished. Growth was measured by the method of Oyama and Eagle(10). The whole medium in which the cells were established was similar to that previously described(11). It contained L-isomers of essential and non-essential amino acids as well as 0.2 mM pyruvate and 5% bovine serum. Serum was omitted from test media. Fatty acid esters were added to media as 2.0% alcoholic solutions containing 2.0% Tween 80 (or Tween 20). For continued passage, cells were grown in T-15 flasks. To transfer to fresh containers, the cells were released from glass surfaces with 0.2 ml of 0.05% crystalline trypsin added directly to medium. An equal quantity of 0.1% trypsin inhibitor (ovomucoid) then was added to stop tryptic activity. The cells were collected by centrifugation and 1/10 to 1/20 of the cells were replanted in 3 ml of serumless medium in T-15 flasks.

Results. Attempts were made initially to grow Walker carcinosarcoma 256 cells in serumless media by appropriately supplementing chemically defined and required amino acids, vitamins, minerals, and glucose. After a series of studies, the medium shown in Table I was derived. In addition to amino acids, vitamins, minerals and glucose, it contains insulin, salmine sulfate, methyl oleate, pyruvate, mucate, folinic acid and lactalysate. The growth promoting effects of the critical constituents (except mucate) were demonstrated as shown in Table II. Each material stimulated growth when added to the otherwise complete medium. In absence of lactalysate, the cells responded favorably to D-galacturonate. However, with inclusion of lactalysate the beneficial effects of D-galacturonate were not observed consistently and apparently were masked or effectively replaced by ingredients of lactalysate. In later studies mucate, available in samples of greater purity than the galacturonate, exhibited similar borderline activity. This compound was retained as a constituent of the medium in view of possible long term effects and to serve in conjunction with purification studies of lactalysate factor(s). D-glucuronate, which has been reported to benefit cells in culture(4), appeared inactive.

TABLE II. Growth Response of Walker Carcinosarcoma 256 to Supplements in Serumless Medium.

Insulin		Salmine sulfate		Methyl oleate		Pyruvate		Lactalysate		Folinic acid	
µg/ml	Cell protein/tube	µg/ml	Cell protein/tube	µg/ml	Cell protein/tube	mM/l	Cell protein/tube	mg/ml	Cell protein/tube	µg/ml	Cell protein/tube
.0	96	.0	100	0	12	.0	78	.0	24	0	86
.001	106	.1	86	1	44	.01	124	.1	26	10^{-4}	86
.05	138	.5	128	3	94	.1	150	.5	102	10^{-3}	114
.1	140	1	124	5	130	.5	162	1	122	10^{-2}	106
.5	172	3	162	8	150	1.0	184	1.5	128	10^{-1}	136
1	192	5	166	10	166	1.5	166	2	136	11	132
2	174	8	192	15	182	2.0	158	3	150	2	102
5	158	10	152	20	156	2.5	132	4	86		
				30	104						
				40	82						

Basal medium in Table I used in these studies.

TABLE III. Effect of Unsaturated Fatty Acid Methyl Esters on Growth of Walker Carcinosarcoma Cells.

Conc. (µg/ml)	Arachidonate	Linolenate	Linoleate	Oleate
	(µg cell protein/tube)			
.0	12	12	12	12
.1	26			
.5	50	40		
1	72	76	46	44
2	68			
3	54	98	136	94
5	10	40	140	130
8				150
10		6	92	166
15			54	182
20			8	156
30				104
40				82

Basal medium in Table I used in these studies. Esters were diluted from alcoholic solutions containing .2% ester and .2% Tween 80.

Lieberman and Ove found insulin to stimulate growth of HeLa and appendix A1 cells (3) and showed that salmine enabled cells to adhere to the glass vessel(12). In the present study both of these substances behaved as growth factors. Zinc tested as a trace metal was inactive.

Pyruvate which promoted growth of isolated Walker carcinosarcoma 256 cells(11) also stimulated growth of cells in the serumless medium.

The requirement for methyl oleate or related compounds is particularly notable. Table III illustrates growth response of Walker carcinosarcoma 256 cells to methyl esters of arachidonic, linolenic, linoleic and oleic acids. Methyl oleate and linoleate were the most effective. Optimal concentration of these unsaturated fatty acids extended over a narrow range, and slightly greater concentrations produced marked inhibitory effects. Tween 80 (polyoxyethylene sorbitan monooleate) possessed a degree of activity but not to an extent which obscured the activity of the fatty acids. Methyl cellulose or Tween 20 (polyoxyethylene sorbitan mono-laurate) when used as suspending agents yielded similar results. Methyl stearate was somewhat active. The methyl esters of caproic, capric, lauric, myristic and palmitic acids did not noticeably stimulate. Esters were employed in these studies instead of the free acids to avoid precipitation of insoluble calcium and

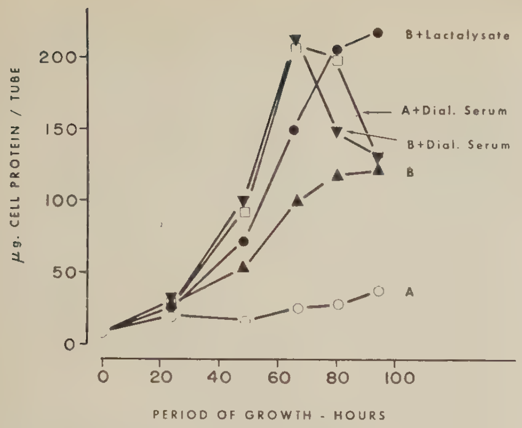

FIG. 1. Growth rates of Walker carcinosarcoma 256 in media with and without additives and serum. A. Medium, Table I, containing 0.2 mM pyruvate, but no other special additives. B. Medium, Table I, containing additives except lactalysate. 5% dialyzed bovine serum included as indicated.

magnesium salts. Ethanol was non-toxic at levels as high as 4 mg/ml.

Lactalysate is the only chemically undefined constituent of the serumless medium. Isolation of the active factor(s) is now in progress in this laboratory. Yeast extract also stimulated though less effectively. Bactopeptone and Bacto-tryptone were inactive.

Fischer reported a sparing effect of folinic acid for a peptone requirement of leukemic cells growing in a medium also containing serum(13). Folinic acid indeed stimulated Walker tumor cells growing in the serumless medium containing lactalysate despite the presence of 2 μg of folic acid per ml. Folinic acid, however, did not replace the requirement for lactalysate.

Variation of amino acids and vitamins, each as a group, and L-glutamine through a concentration range of several-fold showed optimal levels of these groups of nutrients to be that given in Table I.

Catalase, 3, 3′, 5-triiodothyronine, and versene employed by Lieberman and Ove(3) were not included in the present medium because activity for these compounds could not be demonstrated.

The stimulatory effects of insulin, salmine, pyruvate, oleate, folinic acid, and lactalysate recorded for Walker carcinosarcoma 256 cells, were observed to be similarly active for KB, human heart, sarcoma 180, and primary green monkey kidney cells. Evidently these factors have a broad applicability for cell cultures.

Relative growth rates of Walker carcinosarcoma 256 on different media with and without serum are shown in Fig. 1. The simple medium containing only amino acids, vitamins, minerals, glucose, and pyruvate supported little growth. Stimulation resulted from supplementation with chemically defined compounds but lactalysate was required to achieve a growth rate approximating that obtained with serum.

An important criterion of adequacy of a medium is its capacity to support long term growth and repeated subculture. At present the Walker carcinosarcoma 256, KB, human heart, and sarcoma 180 have been carried for 16-20 transfers in serumless medium with no apparent deterioration. A special regimen was not adopted for maintenance of the cultures at most rapid states of proliferation; nevertheless, growth has been such that cultures were routinely subdivided 10-fold every 5-6 days. Evidently, prolonged culture of the cells did not depend upon residual serum protein. Protective or other contributory factors of dialyzed serum were not completely supplied by the present medium for, in addition to a somewhat slower growth rate, inocula as small as 10,000 cells per T-15 flask exhibited poor viability.

As a further criterion for capacity of the serumless medium to support growth, Walker tumor cells were found to grow with a generation time of 25 hours in this medium in cell suspension systems. These suspension cultures were maintained continuously by transfer of 1/8 of the suspension to fresh media at 3-4 day intervals.

Summary. A serumless medium suitable for prolonged growth and repeated subculture of Walker carcinosarcoma 256, sarcoma 180, KB and human heart cells *in vitro* was described. In addition to amino acids, vitamins, minerals and glucose, it contained insulin, salmine sulfate, pyruvate, mucate, methyl oleate, folinic acid and lactalysate. The latter group of materials with exception of mucate was required for maximal growth rates,

comparable to those on media containing dialyzed serum. Suspension cultures of Walker carcinosarcoma 256 were grown with continuous passage in the serumless medium.

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Essential Duration of Parabiosis and Development of Tolerance to Skin Homografts in Mice.* (25798)

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Immunological tolerance of F_1 hybrid skin homografts can be induced in mice of their parent strains after establishing a "F_1 hybrid to parent" parabiosis(1,2). Similarly, female mice of either the A or C57Bl (Subline 1) strains were made tolerant of male skin isografts by placing adult females in parabiosis with the corresponding males(3). Finally, establishment of parabiosis between animals of the Z strain made tolerant of Ce strain tissue by intravenous injection of Ce cells at birth and previously non-conditioned isologous Z mice led to transfer of tolerance. Thus the Z strain animals conditioned only by having been placed in parabiosis with Z animals tolerant of Ce tissue lost their ability to reject homologous skin transplanted from Ce individuals(4). On the basis of these results we postulated that induction of tolerance by the technic of parabiosis might be due to a continuous exchange of all the transplantation antigens, perhaps in the form of intact cells between the 2 parabionts(2,3,4). However, recent experiments performed in this laboratory indicate that length of time during which parabiosis between homologous individuals must be maintained to achieve suppression of the homotransplantation reaction varied with strain of mice used, suggesting that time of foreign antigen exposure and exchange necessary to induce immunological tolerance in a given individual by this method, might perhaps be dependent on degree of histocompatibility difference between the individual and its parabiotic partner, *i.e.*, the more similar the 2 partners, the shorter the length of time in parabiosis required to achieve immunological tolerance.

Our purpose is to document observations bearing on this point and to demonstrate that duration of parabiosis required to produce tolerance is indeed a function of degree of histocompatibility difference between individuals and is also a characteristic of the strains of animals involved regardless of histocompatibility difference.

Method. In one group of experiments mice of the Z[†] strain and F_1 hybrids resulting from

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[†] Z is used to designate mice of C_3H strain originally obtained from Dr. J. J. Bittner's mouse colony.

TABLE I. Essential Duration of Parabiosis in Development of Tolerance to Skin Homografts in Mice.

Parabiosis	Time in parabiosis (days)	Time from separation to grafting (days)	No. of Z parabionts accepting AZF_1 skin grafts/total No. grafted
			%
Z-----AZF_1	7–10	6	1/9 (11)
Idem	23	13	2/7 (29)
"	30	5	7/8 (88)
"	30	20	9/9 (100)
"	40–45	5	23/25 (92)
Z-----Z	25	7	0/13 (0)

the cross between A females and Z males were used. When these animals were approximately 3 months old a "AZ F_1 to Z parent" parabiosis was established. Parabiosis performed was that of the celomic type, as previously described(3). Mice joined together were of approximately the same age and of the same sex. Nine, 23, 30 and 45 days following union, groups of parabiotic pairs were surgically separated under nembutal anesthesia. The peritoneal opening of each parabiont was closed with 5-0 catgut sutures and both muscle and skin layers were approximated with metal wound clips. At varying periods following disruption of the parabiotic union (Table I) the Z partners received a full-thickness abdominal skin graft taken from homologous (A x Z)F_1 hybrid donors. The technic of skin grafting as well as the criteria used to establish acceptance or rejection of the graft were the same as previously described(5). Following grafting of the skin, animals were observed for periods ranging from 5 to 10 months. In a second group parabiosis was established between mice of the Z and Ce strains when these animals were approximately 45 days old. After establishment of the parabiotic union these mice were divided into 2 experimental groups. The members of one group were separated after having been in parabiosis for 27 days and the members of the other group were maintained in parabiosis for a period of 64 days. Eight days following separation mice of both groups received homologous skin grafts taken from adult donors of the same strain as those of their respective partner. After skin grafting, mice were observed for periods ranging from 5 to 10 months to evaluate skin graft survival. For both experiments proper controls consisting of pairs of isologous mice were prepared. After having been kept in a state of parabiosis for periods ranging from 20 to 35 days, members of the control parabiotic pairs were separated and each parabiont was grafted with the corresponding homologous skin, *i.e.*, Z with either (A x Z)F_1 or Ce and Ce with Z.

Results. The results of the first experiment are recorded in Table I. Of 9 Z strain mice which had been in parabiosis with (A x Z)F_1 hybrids for a period of 9 days only, one became tolerant and accepted the (A x Z)F_1 hybrid homologous skin. Of 7 pairs in which parabiosis was discontinued at 23 days, 2 of the Z partners accepted the F_1 hybrid skin homograft. However, when parabiosis was allowed to continue for 30 days, 16 of 17 of the Z partners were found to tolerate (A x Z)F_1 skin grafts. Similar uniformly successful homografts were obtained in the groups maintained in parabiosis for 45 days prior to separation. It will be noted in the Table that time elapsing between disruption of parabiosis and skin grafting did not affect appreciably the results obtained in the different groups.

Table II shows results of second experiment. When parabiosis between Z and Ce mice was disrupted 27 days after establishment, 11 of 12 of the Z animals were tolerant of Ce skin homografts. In contradistinction none of the Ce animals which had been parabiotic partners of the same Z mice accepted homologous Z strain skin grafts. However, when the parabiotic union between Z and Ce animals was disrupted after 64 days tolerance

TABLE II. Essential Duration of Inter-strain Parabiosis on Development of Immunological Tolerance.

Parabiosis	Time in parabiosis (days)	Successful skin homografts*	
		Z partner	Ce partner
Z-----Ce	27	11/12	0/14
Idem	64	11/13	8/13
Z-----Z	35	0/24	
Ce-----Ce	20–40		0/10

* No. of takes/No. of grafted.

of Z skin was present in 8 of 13 Ce parabionts and tolerance of Ce skin was demonstrated in 11 of 13 of the Z parabionts.

In no instance did isologous parabiosis between individuals of either Z or Ce strain result in tolerance of the homologous skin grafts.

Discussion. The results of these 2 experiments are of interest from several points of view. They confirm previous reports demonstrating that acquired tolerance of F_1 hybrid homologous skin grafts can be induced in mice of the parent strain by establishing a "parent to hybrid" parabiosis(1,2). Perhaps more significant is the observation that a high degree of mutual tolerance of skin homografts can be achieved in mice of the Z and Ce strains when joined by this surgical procedure. These results extend to 2 different strains the observations of Rubin(1) that tolerance can be induced between mice of different inbred strains during adult life by placing them in parabiosis.

Results obtained in both experiments indicate that duration of the parabiotic union plays a critical role in determining whether or not tolerance will be induced. For example in the case of Z-----AZ F_1 union, disruption of parabiosis 23 days following operation permitted establishment of tolerance of the AZ F_1 hybrid skin homograft in only 3 of 16 (19%) of Z parabionts. However, when disruption of union was performed 30-45 days after establishment of parabiosis 39 of 42 (93%) of Z parabionts tolerated the corresponding F_1 hybrid skin homografts. Similar findings were obtained in the case of parabiosis between Z and Ce animals. In this instance, the results demonstrate that while 27 days of union was sufficient to abrogate the immunological capacity of most Z parabionts to reject the Ce strain skin graft, this length of time was not sufficient to condition the Ce parabionts to accept Z strain skin homografts. In contradistinction, after 67 days in parabiosis both members of the parabiotic pair became tolerant of the strain of the corresponding parabiont and accepted skin homografts from appropriate donors in almost every instance.

Although difficult of interpretation, as are all findings related to production of a state of tolerance in adult animals, our results add substantially to the accumulating evidence that under certain well defined conditions immunological tolerance can be produced in adult animals. In this regard the procedure of celomic parabiosis seems particularly opportune. The observations support the contention previously submitted that induction of tolerance by parabiosis might be due to a continuous exchange of transplantation antigens between both members of the pair in such amounts as to inhibit capacity for a homograft reaction. This state of non-reactivity which might be considered akin to a state of immunological paralysis could then permit establishment of cells containing all the transplantation antigens in a form capable of replication in the respective hosts, so that upon separation from the parabiotic union, these cells in residence within the recipient host, could maintain the tolerant state between the 2 strains.

It is of interest with respect to the mechanism involved that duration of the parabiotic union appears to be of critical significance. Our observations together with those of Mariani *et al.*(3) indicate that it is more difficult to establish tolerance between Z and AZ F_1 animals than between male and female animals of either the A or C57 Bl (Subline 1) strains by the technic of parabiosis. This conclusion is drawn since it took 30-45 days of parabiotic union to establish tolerance in the Z----AZ F_1 relationship and no more than 5-10 days of parabiosis to establish tolerance between males and females within the same strain.

The studies employing the relatively closely related Z and Ce strain animals (Rubin, personal communication) establish another point. Different strains of mice have different susceptibility to development of acquired tolerance during adult life. For example, in these experiments the Z animals were regularly made tolerant by remaining only 27 days in parabiosis with Ce individuals, whereas the Ce animals required a period of more than 60 days before tolerance was uniformly induced from the same parabiotic union.

Whatever the final explanation, it seems clear that immunological tolerance can be induced in adult animals. It also seems clear that the required antigenic dosage for development of tolerance is very critical and thus the length of time during which homologous parabiosis need be maintained to induce tolerance may be a function of this dosage, which in turn is at least partially consequent to degree of histocompatibility difference between strains. At any rate, length of time during which parabiosis must be maintained to induce immunological tolerance depends upon the strains of mice joined in parabiosis. This observation is strikingly similar to previous observations indicating differences among strains of mice as to capacity of developing classical immunological tolerance when splenic cells are injected intravenously during the neonatal period as well as differences in capacity of donor cells from various strains to induce this phenomenon(5).

Summary. 1. Parabiosis between Z animals and AZ F_1 hybrid animals results in immunological tolerance of the former strain to the latter. 2. When parabiotic union between Z and AZ F_1 animals is maintained less than 30 days a tolerant state is not regularly produced, but when maintained 30 days or more, tolerance of Z for AZ F_1 hybrid skin grafts is the rule. 3. Tolerance between Z and Ce strain mice may be produced in adult life by maintenance of a parabiotic union between members of these 2 strains. 4. Under these circumstances Z mice more readily become tolerant to the strain of the parabiotic partner than do Ce strain mice. 5. Implications of these findings to the theory of immunological tolerance are discussed.

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Penetration of Radioactive Phosphorus into Normal and Shocked Rats' Brain.* (25799)

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Traumatic shock is accompanied by changes in enzyme activities of liver, heart and brain. Strawitz and Hift(1) and Packer, Michaelis and Martin(2) have demonstrated uncoupling of oxidative phosphorylation, as well as shifts in concentrations of phosphate, sodium, potassium and calcium, accompanied by swelling of mitochondria in shock. The intracellular changes suggested that the permeability of some tissues might change in shock, and that in particular the blood brain barrier might be affected. This possibility was tested with an autoradiographic method. When the Masson trichrome stain, as modified by Goldner, was used on brains of normal and shocked rats‡ no significant differences could be found.

Method. Young adult Wistar rats weighing 200-300 g were injected intraperitoneally with P^{32} inorganic phosphate, approximately 35 μc per 100 g body weight. Shock was induced within 10 minutes after injection with the drumming method(3) in an apparatus consisting of 4 drums driven by one motor.

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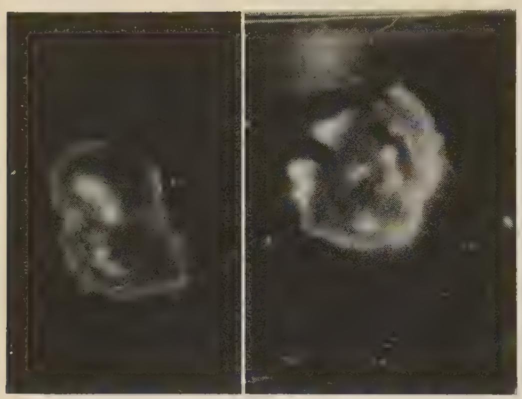

FIG. 1. Autoradiographs of sagittal sections of rats' brain after intraper. inj. of P^{32} phosphate.

The inner diameter of the drums was 37.4 cm. Each drum was fitted with 2 prisms with bases fixed peripherally facing each other, on the same radius. Fore and hind limbs of the animals were tied with adhesive tape. The rats were tumbled for 500 turns during 12.5 minutes. They were then unfettered and held in individual cages for 2 hours. During this period shock developed, with prostration, shallow breathing and, in most cases, cyanosis. Both experimental and control animals were decapitated having been exposed to the radioactive phosphate for the same length of time. Their brains were excised and fixed in 10% formalin. Sagittal sections, 50 μ thick, were made and exposed on Kodak No Screen X-Ray film for 4 weeks, in a refrigerator. The films were developed and radioactivity was examined. Eight experiments were done with 13 controls and 26 tumbled rats.

Result. The photographs (Fig. 1) show typical samples of autoradiographs of normal and tumbled rats' brains. In normal brains radioactivity was noted with clear outlines around the regions which were bathed by cerebrospinal fluid, such as walls of the ventricles and surface of the cerebral cortex. In tumbled rats, diffuse penetration of P^{32} into the brain tissues was found.

Discussion. Borell and Örström(4) reported that after intraperitoneal injection of radioactive phosphate into rats and rabbits, the choroid plexus took up the isotope 10 to 80 times more rapidly than did nervous tissue. Bakay(5) observed that after intracisternal injection of P^{32} the brain took up large amounts quite rapidly. These reports indicate that the brain has an inherently rapid phosphate metabolism, but that penetration of phosphate from the blood stream into it is normally severely delayed. Bakay (6) suggested that radioactive phosphate enters the brain *via* the cerebrospinal fluid after it has passed the blood-cerebrospinal fluid barrier during initial phase of absorption. The feature of this phase is a large deposition of phosphorus in the surface area, and presumably because of a direct passage of the phosphate through the blood brain barrier by transcapillary exchange, the latter phase of absorption shows a very gradual increase of P^{32} in the whole brain.

Summary. The clear outline in our controls around regions bathed by cerebrospinal fluid may be due to P^{32} after it had passed through the blood cerebrospinal fluid barrier. The diffuse and widespread penetration of P^{32} into brains of tumbled rats indicates alteration of permeability caused by damage at the blood brain barrier. This penetration can not be seen with usual stain technics nor is it due to dispersion following extensive petechial hemorrhage within the brain substance.

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Effect of Daily Exposure to 15 r γ Radiation on Susceptibility of Mice to Experimental Infection.* (25800)

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In earlier experiments(1) mice were exposed continuously to gamma radiation at 3 different levels of intensity: approximately 128, 69, and 34 r/day, respectively. After various periods of irradiation their susceptibility to bacterial infection was determined by intraperitoneal challenge with graded inocula (10-fold dilutions) of *Pseudomonas aeruginosa*. The results showed that increased susceptibility to this infection was related to dose rate rather than total amount of radiation accumulated. At the lowest rate, 34 r/day, no significant increase was demonstrated in 2 experiments on mice which had accumulated 2140 r during 9 weeks exposure. Despite that finding, dose rate in the present experiments was reduced to 15 r/day, but the challenge inocula (of the same test microorganism) were graded by less than 10-fold dilution in an atempt to detect smaller changes in susceptibility than were demonstrable in the previous experiments.

Materials and methods. Mice. A total of 895 CF-1 females were used, approximately half of them irradiated and half controls. In one experiment they were 4 weeks old, in all others 10 weeks old, when irradiation was begun. They were housed in groups of 10 in Lucite cages measuring 7½ x 12 x 7 inches covered with perforated aluminum tops. Food pellets were available at all times. Tap water was supplied in sterilized bottles which were changed every day. Cages were changed and disinfected once a week. *Irradiation.* The mice received approximately 15 r/day 6 days a week. Exposure time was 7 hours at first, gradually lengthened to 7 hours 21 minutes as the Co^{60} source decayed. Between exposure periods both irradiated and control mice were kept in the same room, one week in a room reserved for uninoculated mice, the next week in the exposure room with the source shielded. This was done so that irradiated and control mice should live as much of the time as possible in the same environment. No deaths occurred among irradiated or control mice before challenge. The longest exposure period was 15 weeks because it was necessary to terminate experiments at that time. *Exposure.* Mice were exposed in an air conditioned room in the sub-basement of the Argonne Cancer Research Hospital.[†] Cages were placed on a wooden rack with their long sides facing a nominal 10-curie Co^{60} source, their centers all 124 cm from it. The dose rate was based on the assumption that distribution of the mice was random within each cage and disregarded the shielding effect of the mice on each other. *Dose measurements* were made with a Victoreen ionization chamber (calibrated by the National Bureau of Standards) placed in the center of one of the Lucite mouse cages with its aluminum top in place. The ionization chamber was covered with a close-fitting 4-mm Lucite cap of sufficient thickness to provide electronic equilibrium for cobalt-60 gamma rays. Accuracy of these measurements was estimated to be $\pm$ 3%. *Challenge inoculations.* Each challenge inoculation included unirradiated controls from the same shipment of mice. The test microorganism was a streptomycin-resistant strain of *Pseudomonas aeruginosa* used in this laboratory for several years to challenge mice exposed to acute X-radiation(2,3) and to continuous gamma radiation(1). Virulence has been maintained by weekly passage through mice. *Preparation of inocula.* An 18-hour culture was washed off an agar plate, suspended in 5 ml saline and shaken 15 minutes on a rotator in a flask containing glass beads to disperse

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[†] Operated by Univ. of Chicago for U. S. Atomic Energy Commission.

TABLE I. Total Leucocyte Counts (Geometric Means) on Mice Exposed to 15 r/Day.

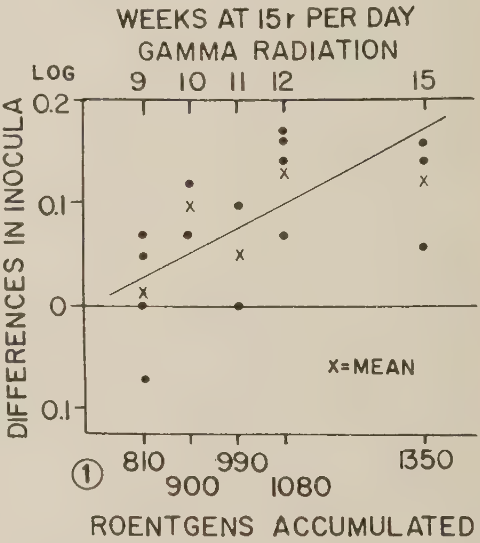
Exposure, wk	Radiation accumulated, r	Leucocytes/mm^3
0	0	8150
1	90	6150
2	180	6000
3–4	270–360	5150
5–6	450–540	5100
7	630	4800
9–10	810–900	2700
11	990	2650
12–13	1080–1170	2800
15	1350	2200

any clumps of bacteria. The suspension was diluted to a standard density (10^9 per ml) by means of a Coleman colorimeter (filter 430 mm). Dilutions were made to provide inocula containing approximately 2.0, 1.2 and 1.0 x 10^8 (and in a few experiments 10^7) which were injected intraperitoneally in 0.5 ml volume into 5-20 mice each. The numbers were checked by plating in quadruplicate 0.1 ml samples of appropriate dilutions of the bacterial suspensions. *White blood counts.* Total leucocyte counts were made on 242 mice by customary methods on blood obtained from a tail vein. Their geometric means are shown in Table I.‡

Results. Almost all deaths occurred within 48 hours after challenge. Although 13 mice died later, autopsy cultures of heart's blood and spleen showed only 6 of them to have died with *Ps. aeruginosa* bacteremia. The other 7, therefore, were excluded from the mortality data.

From the results of each challenge inoculation, the LD_{50} for irradiated and control mice was determined by probit analysis. The difference between LD_{50} of each irradiated group and its controls is plotted (in tenths of a log) against the duration of their exposure, *i.e.*, accumulated radiation (Fig. 1). The mean of these differences (ratios) for each exposure time is also plotted. Points for the regression line were computed from the means by the method of least squares. It shows that a slight but progressive increase in susceptibility to experimental infection resulted from prolonged daily exposure to 15 r. Between 9th and 15th weeks, the period covered, this increase was calculated to be .029 log of inoculum per 100 r gamma radiation accumulated.

Comparison with effect of single exposure to X-radiation. In earlier experiments(3), mice were subjected to 300, 400, and 500 r X-radiation in a single acute exposure and challenged 5 days later by intraperitoneal inoculation with the same strain of *Ps. aeruginosa.* LD_{50}s



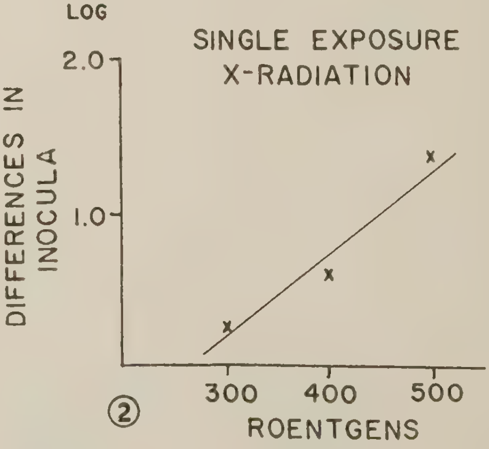


FIG. 1. Changes in susceptibility to challenge inoculation plotted as differences in LD_{50}'s of irradiated and control mice.

FIG. 2. For comparison: effect of single acute exposures to X-radiation. Challenge 5 days post-irradiation with same strain of *Pseudomonas aeruginosa.*

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‡ Leucocyte counts were made by Miss Betty Wolfe.

were computed by the methods described above. The means of the differences between those of irradiated and control mice are plotted (as *whole* logs) in Fig. 2. The slope shows how much greater was the increase in susceptibility to the challenge inoculations produced by each increment of 100 r acute X-radiation, approximately .54 log of inoculum per 100 r.

Discussion. The differences in LD_{50}s of *Ps. aeruginosa* for irradiated and control mice in each challenge inoculation indicate a slight but progressive increase in susceptibility to this bacterial infection from the 9th through the 15th weeks of exposure to 15 r/day. The change was too small to have been demonstrable by use of 10-fold gradations of inocula as used in our previous experiments on mice exposed to 34 r/day(1). At 9 weeks, the shortest exposure time, the effect on susceptibility was so slight as to raise the question whether any change at all could have been detected earlier.

After 15 weeks' irradiation (total accumulation 1350 r) the increase in susceptibility, while still small, was clearly demonstrable. It is unfortunate that exposures could not have been prolonged beyond that time. Comparison of this effect of chronic γ irradiation with that produced by a single exposure shows that an accumulation of 1350 r (at 15 r a day) increased susceptibility to the challenge inoculations much less than did an acute exposure to 300 r X-radiation.

The effect on total leucocyte counts was about the same. The geometric mean of the white counts on 5th day following exposure to 300 r was 2400(1). After accumulating 1350 r (at 15 r a day) it was 2200.

Summary. 1) CF-1 female mice were exposed to approximately 15 r a day radiation for 9-15 weeks and challenged at intervals by intraperitoneal inoculation with graded doses of *Pseudomonas aeruginosa.* Comparison of LD_{50} of irradiated and control mice in each challenge showed that between 9th and 15th weeks a slight but demonstrable increase in susceptibility to experimental infection had occurred. 2) The increase caused by accumulation of 1350 r during 15 weeks exposure was, however, very much less than that resulting from a single acute exposure to 300 r X-radiation.

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Induction of Lactation and Mammary Growth by Pituitary Grafts in Intact and Hypophysectomized Rats.*† (25801)

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Recent investigations suggest that the hypothalamus normally inhibits prolactin secretion by the AP, and that severance of the pituitary from its cranial connections results in increased production of this hormone. Desclin(1-2) and Everett(3) reported that autotransplantation of the pituitary to the kidney capsule of the rat maintained functional corpora lutea for prolonged periods of time. Eckles *et al.*(4) noted lactation in several

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women with metastic breast cancer after pituitary stalk-section, and Loeb and Kirtz(5) and Muhlbock and Boot(6) reported that subcutaneous isografts of pituitaries into intact mice induced mammary growth. It was of interest to see whether pituitaries grafted to the kidney capsule of intact or hypophysectomized rats, after estrogen priming, could produce sufficient prolactin to induce secretion and maintain or increase mammary growth.

Methods. Sixty mature virgin, female Carworth rats, weighing 180-210 g each, were injected subcutaneously with 10 μg estradiol in corn oil once daily for 10 days to partially develop their mammary glands. In all except 10 controls, pituitaries from similar donor rats were implanted underneath the kidney capsule on days 1, 5 or 7 of estradiol treatment. Twenty-four of these rats were hypophysectomized on day 11 by the parapharyngeal approach, and these and all other rats were given no further treatment for the next 5 days. On the 16th day the rats were killed, and the right inguinal mammary glands, pituitary graft, adrenals, thymus, ovaries and uterus were removed and prepared for microscopic examination by standard histological technics. The latter 4 organs were also weighed. Ratings of 0-3 were assigned for mammary development and lactation in each case, depending on extent of mammary lobulo-alveolar development and distention of alveoli with secretion.

Results. In previous experiments it was established that rats at the end of 10 days' injection with 10 μg estradiol daily developed considerable lobulo-alveolar tissue (avg rating = 1.5), and that absence of further treatment for the following 5 days resulted in regression of the mammary glands to essentially a bare duct system(7). This was also evident in the present controls. By contrast, in 24 intact rats with pituitary grafts, lactation was initiated in 20 and mammary lobulo-alveolar structure was maintained or increased in 23 rats. Pituitary implants made on day 1 induced lactation in all 7 rats and produced greater mammary growth than in the 17 rats implanted on day 7 (Table I). In 26 hypophysectomized rats with pituitary grafts, mammary secretion was initiated in only 8 and lobulo-alveolar tissue was maintained in 12 of 26 animals. Mammary glands of the remaining hypophysectomized rats were atrophied and contained mainly ducts, as in the

TABLE I. Effects of Pituitary Implants in Intact and Hypophysectomized Rats on Mammary Glands and Several Organ Weights.

Treatment	No. of rats	No. with secretion	No. with LA*	Avg body wt (g)		Avg organ wt (mg)/100 g body wt			
				Initial	Final	Adrenals	Thymus	Ovaries	Uterus
				Intact rats					
Controls, saline	10	0	0	210	218	27.5 ± 1.5‡	99.9 ± 8.8	26.1 ± 1.8	207.1 ± 19.0
Pit. implant on day 1	7	7 (1.0)†	7 (2.5)†	202	224	30.8 ± 2.8	38.7 ± 17.8	25.6 ± 5.2	191.2 ± 19.3
Idem 7	17	13 (0.9)	16 (1.8)	195	205	30.2 ± 2.4	76.1 ± 14.6	21.5 ± 2.8	168.7 ± 19.8
				Hypophysectomized rats					
” 1	12	2 (0.1)	4 (0.7)	187	174	20.7 ± 2.2	70.0 ± 11.6	22.4 ± 2.0	156.0 ± 10.4
” 5	14	6 (0.5)	8 (1.6)	189	174	16.1 ± 1.1	62.1 ± 7.2	17.2 ± 1.6	156.0 ± 9.7

* LA = lobulo-alveolar development.

† Avg rating = $\frac{\text{Sum of individual ratings (0-3)}}{\text{Total No. of rats in group}}$.

‡ Stand. error of mean.

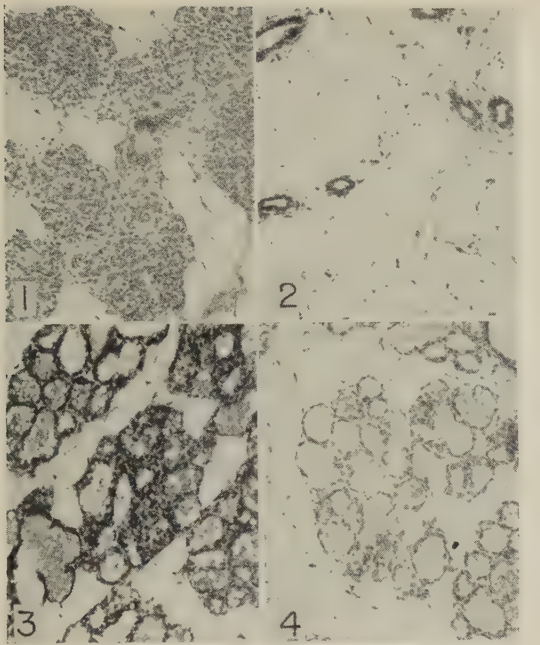

FIG. 1-4. Histological sections of mammary tissues from estrogen-primed rats with and without pituitary grafts: (1) From non-grafted rat on 10th day of estradiol treatment. Note development of lobulo-alveolar system but no secretion. (2) From non-grafted rat on day 16. Note regression to bare duct system. (3) From intact rat grafted with pituitary on day 1, showing well-developed alveoli extended with secretion. (4) From hypophysectomized rat grafted with pituitary on day 5, also showing secretion and development of alveoli. ×72.

controls. Representative microphotographs of mammary gland sections are shown in Fig. 1-4.

The pituitary grafts appeared healthy and well vascularized in most intact and hypophysectomized rats. In a few hypophysectomized rats, grafts could not be recovered from the kidney capsule and had apparently slipped out. However, the presence of heavily luteinized ovaries in these rats suggested that the pituitaries were functional. Everett(3) has similarly described pituitary transplants to the kidney capsule of rats which slipped out and became functional elsewhere. The ovaries of both intact and hypophysectomized rats with grafts showed a predominance of healthy corpora lutea with few or no small follicles. The corpora lutea were usually larger in the intact than in the hypophysectomized rats. Ovaries of 2 intact rats implanted on day 7 had large follicles and only small corpora lutea, and these showed no mammary secretion. Adrenal weights were significantly lower in hypophysectomized rats than in intact rats, and appeared to be atrophied upon microscopic examination.

Discussion. These data show that pituitary isografts to the kidney capsule of rats can initiate secretion and maintain or extend mammary growth developed by prior estradiol treatment. Preliminary experiments also indicate that in intact postpartum rats after litter removal, pituitary isografts to the kidney capsule can inhibit mammary involution (Nicoll and Meites, unpublished). Mammary growth and secretion were much more pronounced in grafted intact than in grafted hypophysectomized rats, and might have been due to a relative deficiency of ACTH, TSH and STH secretion by the latter animals. Secretion of these 3 hormones as well as FSH and LH are markedly reduced when the pituitary is removed from its normal cranial site (8). Adrenal cortical hormones as well as prolactin are required to initiate lactation in hypophysectomized rats(9), and adrenals of the hypophysectomized rats with pituitary grafts in the present experiment were notably reduced in size.

The present results, in agreement with others, indicate that the transplanted pituitary does not need CNS stimulus to secrete prolactin, and actually produces more prolactin when its connection to the hypothalamus is severed. We have recently reported that many agents can induce mammary growth and secretion in estrogen-primed rats, including adrenergic and cholinergic drugs(7,10), CNS stimulators and inhibitors(11), various tranquilizing drugs(11,12), electrical stimulation of uterine cervix(13), nonspecific stresses(14) and rat hypothalamic extract‡(15). Some of these, such as reserpine, chlorpromazine or electrical stimulation of the uterine cervix may act by removing CNS inhibition to prolactin secretion. Others may act directly on the anterior pituitary to remove CNS inhibition or by some other mechanism. It remains to be established that a prolactin-inhibitory neuro-hormone is actually present in the CNS.

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‡ Positive results with hypothalamic extracts suggest that the CNS may contain a prolactin-releasing center.

Summary. 1. Sixty mature female rats were injected with 10 μg estradiol daily for 10 days to develop their mammary glands. On days 1, 5 or 7, all except 10 control rats were each implanted underneath the kidney capsule with a pituitary from a similar untreated donor rat; on day 11, 26 implanted rats were hypophysectomized. All rats were killed on day 16. 2. Control mammary glands regressed to a bare duct system and showed no secretion, whereas 23 of 24 intact rats with pituitary grafts and 12 of 26 hypophysectomized rats with grafts showed mammary secretion and/or lobule-alveolar development. Most rats with pituitary implants had ovaries with large corpora lutea and few or no follicles. These results demonstrate that grafted pituitaries in the rat secrete greater than normal amounts of prolactin.

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Chemotherapy of Sarcoma 180 by Combinations of DL-Glyceraldehyde with 6-Thioguanine or with Azaserine and 6-Chloropurine.* (25802)

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Glyceraldehyde has been reported in a number of instances to be a potent glycolytic inhibitor; this was recently reviewed briefly by Gold(1). The mechanism of inhibition was shown by Lardy *et al.*(2) to be due to condensation of L-glyceraldehyde with dihydroxyacetone phosphate to yield L-sorbose-l-phosphate, which subsequently interfered with the hexokinase reaction. In view of this inhibition and of the knowledge that nucleotide formation requires carbohydrate as well as purine or pyrimidine moieties, the present study was designed to test anti-neoplastic properties of combinations of inhibitors of these systems.

Methods. Experiments were performed on 9 to 11-week-old female Ha/ICR Swiss mice (A. R. Schmidt Co.). Tumor transplantation was carried out by withdrawing ascites fluid from a donor mouse bearing a 7-day tumor growth. The fluid was centrifuged for 2 minutes in a clinical centrifuge (1600 x g), supernatant peritoneal fluid was decanted, cells were diluted 1:10 with isotonic saline, and 0.1 ml (approx. 2 x 10^6 ascites cells) of cell suspension was inoculated into each animal. Mice were distributed into groups of comparable weight and maintained during experi-

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TABLE I. Effects of DL-Glyceraldehyde and 6-Thioguanine on Survival Time of Sarcoma 180 Tumor-Bearing Mice.

Daily dose (mg/kg)				
Glyceraldehyde	Thioguanine	Avg survival (days)	No. 50-day survivors*	Avg wt (g)†
0	.0	11.8	0/34	+4.4
0	1.0	20.5	0/25	+4.8
0	2.0	19.5	0/10	+4.0
0	3.0	12.5	”	+2.7
200	.0	19.9	”	+1.5
400	.0	26.6	3/25	− .2
600	.0	29.6	3/20	+ .1
800	.0	18.7	0/10	− .4
200	1.0	28.5	2/10 (1)	− .7
400	1.0	33.0	4/15 (3)	− .3
600	1.0	29.9	5/19 (1)	−1.6
400	2.0	29.0	1/10	− .7
400	3.0	25.7	”	−1.7

* Mice surviving over 50 days were calculated as 50-day survivors in determination of avg survival time. Figures in parentheses indicate No. of complete regressions.

† Avg wt change from onset to termination of drug treatment.

ments on Rockland rat chow pellets and water *ad lib*. Drugs were injected intraperitoneally in isotonic saline; thioguanine was solubilized in dilute sodium hydroxide and neutralized to pH 7-8 with hydrochloric acid. Therapy was initiated 24 hours after tumor implantation and was continued once daily for 6 consecutive days (combination treatments given simultaneously). Tumor-bearing controls received injections of isotonic saline. Animals were weighed daily during treatments, and weight change from onset of therapy was used as an indication of drug toxicity. Survival time was used as the criterion of tumor inhibition. Mice surviving over 50 days and tumor-free animals were calculated as 50-day survivors in determination of average survival time. Complete regression of tumor growth was established by 2 criteria: drug-treated animals were maintained for 75 days to allow any surviving ascites cells to become evident; ascites tumor-free animals were then autopsied for presence of solid tumors in the peritoneal cavity. All gross nodules were examined histologically for presence of malignant cells.

Results. The effects of DL-glyceraldehyde and combinations of glyceraldehyde and thioguanine on survival time of mice bearing Sarcoma 180 are shown in Table I. Glyceraldehyde showed demonstrable effects at all dose levels tested; optimum dose was between 400-600 mg/kg of body weight. Addition of thioguanine to glyceraldehyde treatments appeared to increase slightly the effectiveness.

Table II shows the results obtained employing a combination of glyceraldehyde with azaserine and chloropurine. The effect produced by simultaneous administration of these agents was greater than that obtained at optimum doses of glyceraldehyde (Table I). Eleven out of 15 treated animals survived a minimum of 50 days; of these, 2 were complete regressions of tumor growth.

At a level of 400 mg/kg, glyceraldehyde produced a doubling of survival time of C3H mice bearing Hepatoma 134 cells, but it was without effect on survival of AKR mice bearing Mecca lymphosarcoma cells.

Discussion. The present results with glyceraldehyde are in direct contrast to previously reported(3) ineffectivenes of this compound against solid implants of the same tumor. Perhaps sufficient concentration of the inhibitor did not reach the subcutaneous implant or different strains of Sarcoma 180 were involved in the 2 experiments. The marked prolongation of survival time noted supported

TABLE II. Effects of DL-Glyceraldehyde in Combination with Azaserine and 6-Chloropurine on Survival Time of Sarcoma 180 Tumor-Bearing Mice.

Daily dose (mg/kg)					
Glyceraldehyde	Azaserine	Chloropurine	Avg survival (days)	No. 50-day survivors*	Avg wt (g)†
0	.0	0	10.9	0/14	+2.9
400	.0	0	25.6	0/10	+ .0
0	.2	40	30.2	3/15 (1)	+3.6
400	.2	40	44.5	11/15 (2)	− .9

* Mice surviving over 50 days were calculated as 50-day survivors in determination of avg survival time. Figures in parentheses indicate No. of complete regressions.

† Avg wt change from onset to termination of drug treatment.

the hypothesis that interference with glycolysis would inhibit neoplasia.

Combinations of glyceraldehyde, azaserine, and chloropurine were more effective than once daily treatments with glyceraldehyde or with azaserine and chloropurine. The results obtained were comparable to those observed using the same doses of azaserine and chloropurine given twice daily(4). The ability to demonstrate therapeutic synergy in the present system was limited by the potency of azaserine and chloropurine.

Summary. DL-glyceraldehyde prolonged survival time of mice bearing Sarcoma 180 or Hepatoma 134 ascites cells. Mecca lymphosarcoma ascites cells proved resistant to this agent. Combination of glyceraldehyde, azaserine, and chloropurine in the therapy of Sarcoma 180 produced a greater effect than that found using glyceraldehyde alone or with the dose level of azaserine and chloropurine employed.

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Site of Intracellular Antigen Production by Myxoviruses.* (25803)

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Application of fluorescent antibody technics to the study of multiplication of myxoviruses has suggested that the nucleoprotein component of influenza(1,2) and fowl plague(3, 4) viruses is produced within the nuclei of infected cells. The most complete study of this phenomenon is that by Breitenfeld and Schafer(3). From their experiments with chicken embryo fibroblasts infected with fowl plague virus they concluded that viral hemagglutinin is produced in the cytoplasm, whereas the nucleoprotein component of the virus particle is produced within the nucleus and later migrates to the periphery of the cell where it is combined with hemagglutinin to form complete, infectious virus particles. In our studies of the effect of Sendai virus on cells it was found that intranuclear antigen was not demonstrable by use of fluorescent antibody. Since this appeared to represent an important difference in characteristics of multiplication of viruses within the myxovirus group, the phenomenon was studied further. This paper compares the multiplication cycles of Sendai, mumps, and Newcastle disease viruses with that of influenza A virus in regard to intracellular site of antigen production.

Materials and methods. Viruses. The PR-8 strain of influenza A, the Roakin strain of Newcastle disease virus (NDV), and the Enders strain of mumps virus were used. These were egg-adapted lines of virus cultivated by standard methods in the allantoic sac of chicken embryos. The Dunai strain of mumps virus(5) adapted to serial cultivation in human conjunctiva cells was used as virus from the 8th tissue culture passage. The Sendai strain of para-influenza I virus was cultivated in the allantoic sac of chicken embryos(5). A line of Sendai virus adapted to serial cultivation in human conjunctiva cells was used as virus from the 7th tissue culture passage. *Cell cultures.* Primary cultures of chick embryo lung cells were prepared from lungs of 11-day-old embryos(6). They were grown in Scherer's medium with addition of 5% chick embryo extract and 15% horse serum and were maintained in this medium after inoculation. Human conjunctiva cells (Chang) were grown in serial culture in Eagle's basal medium containing 20% horse serum and after inoculation

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were maintained in Scherer's medium containing 5% horse serum. For examination by fluorescence microscopy cells were grown on cover-glasses in tubes with flattened sides. Before inoculation the medium was removed and the cells washed with a balanced salt solution. Cells were exposed to the inoculum, containing the appropriate quantity of virus in 1.0 ml of culture maintenance medium, for 30 min. at room temperature, after which the inoculum was removed, the cell sheet was washed with balanced saline, and 1.0 ml of maintenance medium added. Timing of virus growth cycles was begun 15 min after addition of virus inoculum. At selected intervals the medium was removed from cultures, and the cells were washed twice with balanced saline. Cover-glasses were removed, fixed in acetone for 10 min, then dried in air. Cell preparations were stored in a desiccator at 4°C until time of microscopic study. *Antisera.* To produce antiserum against influenza and Sendai viruses mice were given an intranasal instillation of diluted infected allantoic fluid. An inoculum was selected that resulted in pulmonary infection and about 10% mortality. Three weeks after inoculation the mice were given an intraperitoneal injection of 0.05 ml of a saline extract of 20% infected mouse lung homogenate. This was repeated one week later and the mice were exsanguinated 1 week after the second booster injection. Antisera were produced against Sendai and mumps viruses in 800 g guinea pigs by giving the animals an intranasal instillation of 0.5 ml of undiluted infected allantoic fluid. Guinea pigs given mumps virus were bled 2 weeks after inoculation and those given Sendai virus were bled 3 weeks after inoculation. Antiserum against NDV was produced by infecting adult chickens *via* the intranasal route with an inoculum that caused obvious illness in most chickens and an occasional death. Chickens were bled 3 weeks after inoculation. Except for chicken sera, all sera were tested by complement-fixation for antibody content against the S (nucleoprotein) and V (hemagglutinin protein) antigens of the appropriate virus. Sera selected for conjugation with fluorescein were ones with good levels of antibody against both viral antigens. Gamma globulin was precipitated from antisera with methanol and conjugated with fluorescein isothiocyanate (7). Conjugated preparations were absorbed with mouse liver powder and chick embryo powder. *Microscopy.* Infected cells on cover slips were stained with fluorescent antibody using the direct method(8). Controls consisted of uninfected cells treated with specific conjugate, infected cells treated with normal serum, and, on certain occasions, infected cells pre-treated with unlabeled antibody prior to staining with labeled antibody. Slides were examined using a 200 watt high-pressure mercury-vapor lamp UV light source and a BG 12 excitor filter. For observation a Wratten 3 barrier filter was used. Photographs were made using a Wratten K2 barrier filter, Kodak Plus-X film, and an exposure time of 2 min.

Results. Influenza virus. Watson and Coons(1) noted a nuclear phase in multiplication of influenza virus in the chicken embryo and similar observations were made by Liu (2) who studied the infected respiratory epithelium of ferrets. No detailed study employing fluorescent antibody and following development of the PR8 strain of influenza A virus in a single cycle of multiplication had been published, however, so it was necessary to examine the cycle of influenza virus to be able to compare it with cycles of NDV, mumps and Sendai viruses.

Cultures of chicken embryo lung cells were inoculated with the PR8 strain of influenza A virus using a multiplicity of approximately 10 egg-infectivity doses (EID_{50}) per cell. Observations were made at 1, 2, 4, 6, 8, 12, 20, and 26 hr after inoculation. No specific fluorescence was noted in the cells until 6 hr, at which time bright yellow granules were seen in the nuclei of some cells. The nuclear membrane of such cells appeared as a fine yellow line; no specific fluorescence was noted in the cytoplasm. At 8 hr in most cells the nucleus fluoresced as a solid yellow area, and a few small granules had appeared in the cytoplasm (Fig. 1A). Cytoplasmic fluorescence increased until at 20 hr both nucleus and cytoplasm contained very fine yellow particles

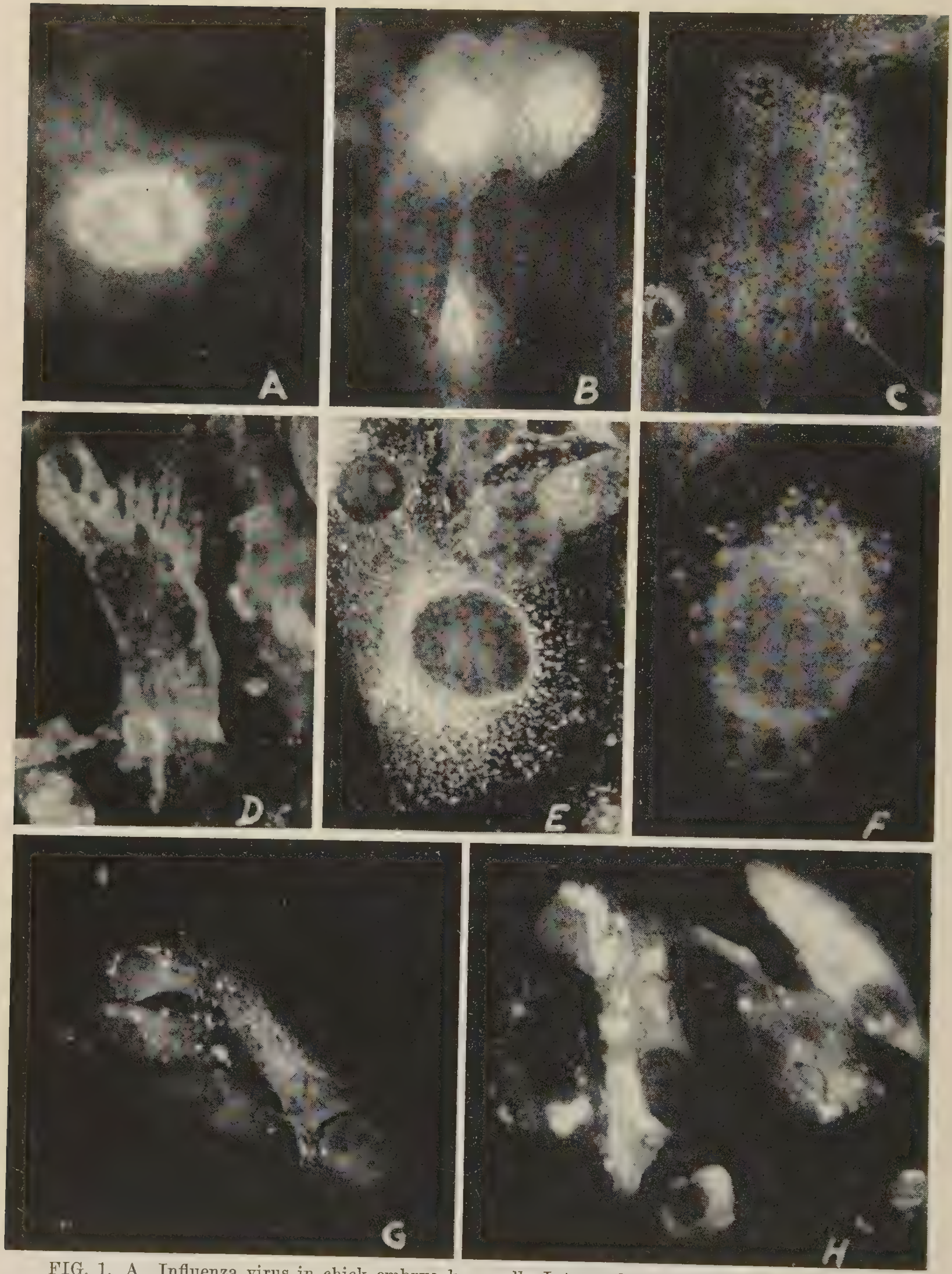

FIG. 1. A. Influenza virus in chick embryo lung cell. Intranuclear antigen at 8 hr. ×1000. B. Influenza virus in chick embryo lung cells at 20 hr. ×750. C. Sendai virus in chick embryo lung cell at 8 hr. ×450. D. Sendai virus in chick embryo lung cell at 26 hr. ×450. E. Sendai virus in human conjunctiva cells at 12 hr. ×450. F. NDV in chick embryo lung cell at 6 hr.

which imparted a dusty appearance to the cells and made it difficult in many cells to differentiate nucleus and cytoplasm (Fig. 1B). At 26 hr the nucleus had become relatively free of fluorescent material, but the cytoplasm contained many fine granules and a few larger fluorescent masses. Infectivity and hemagglutination titrations carried out on fluids from these cultures suggested that this represented a complete multiplication cycle. Measurable hemagglutinin levels were not reached in the 26 hr observation period but infectious virus release was first detectable at 12 hr and titers in the fluid had increased 200-fold by 26 hr.

When chicken embryo lung cells were inoculated with one EID_{50}/cell only about 10% of the cells produced antigen in the first cycle, but there was no essential difference in evolution of the pattern of antigen distribution from that seen with larger inocula.

Sendai virus. Chicken embryo lung cells were inoculated with 1000 EID_{50}/cell of Sendai virus previously maintained in the allantoic sac of the chicken embryo and cell preparations were collected for observation at 1, 2, 4, 6, 8, 12, 20 and 26 hr. Specific viral antigen was first visible in the cells at 2 hr as fine granules distributed throughout the cytoplasm. At 4, 6, and 8 hr (Fig. 1C) the cytoplasmic antigen steadily increased in quantity. At 12 hr some of the cells showed definite cytopathic effect. By 26 hr most cells contained large masses of cytoplasmic antigen (Fig. 1D) and many were rounded and contracted except for several long, fine cytoplasmic processes extending from each cell. The nucleus could be seen as a non-fluorescent area throughout the multiplication cycle. Hemagglutination and infectivity titrations performed on the fluids from these cultures suggested that the cycle of egg-adapted Sendai virus in chick embryo lung cells was largely incomplete. Very small amounts of infectious virus appeared in the culture fluid at 12 hr, but there was no further increase until 26 hr when cell degeneration was prominent, and even then titers of infectious virus reached no higher than $10^{-2.75}$. Hemagglutinin titer was 1:8 at 20 hr and 1:16 at 26 hr.

Study of a multiplication cycle of Sendai virus yielding complete infectious virus was made employing a virus line adapted to serial culture in human conjunctiva cells. Experiments showed that approximately 100% of cells in a culture of conjunctiva cells could be infected with an inoculum of 7 EID_{50} per cell. In one experiment the virus was diluted so that about 10% of cells were initially infected. After a latent period of 6 hr infected cells were first distinguishable by the presence of 15-20 small brightly fluorescent granules dispersed throughout the cytoplasm. The nucleus appeared as a clear, non-fluorescent area. At 8 hr the number and size of cytoplasmic particles of antigen had approximately doubled over that observed at 6 hr, but there was no change in general distribution of antigen. At 12 hr the cytoplasm was heavily stippled with antigen but the nucleus remained clear (Fig. 1E). By 24 hr the number of infected cells had increased to about 25%, apparently due to a second cycle of multiplication.

When conjunctiva cells were inoculated with 1000 EID_{50} per cell of egg-adapted Sendai virus all cells contained visible antigen at 2 hr after inoculation. At that time cells contained 20-40 small fluorescent particles scattered diffusely throughout the cytoplasm and no antigen was visible in nuclei. The number of cytoplasmic granules steadily increased and by 9 hr some larger masses of antigen had formed. Cytopathic effect was evident at 22 hr in formation of long cytoplasmic processes. At this time the entire cytoplasm was filled with large masses and fine granules of antigen. Throughout the multiplication cycle the nucleus could be distinguished by its lack of specific fluorescence.

In study of Sendai virus in conjunctiva cells it often was noted that cells in various stages of mitosis contained cytoplasmic antigen. Wheelock and Tamm(9) have similarly reported that antigen of influenza A and NDV

×900. G. NDV in chick embryo lung cells at 22 hr. ×450. H. Mumps virus in human conjunctiva cells at 26 hr. ×500. Direct observation suggested strongly that the small amounts of fluorescent material in nuclear area in C-H were due to overlying or underlying cytoplasmic antigen.

can be demonstrated in infected HeLa cells undergoing mitosis, although in fowl plague infection of chicken macrophages Franklin found cells in mitosis to be notably free of antigen(4).

Newcastle disease virus. Chick embryo lung cell cultures inoculated with 100 EID_{50}/cell were studied at 1, 2, 4, 6, 8, 12, and 22 hr after inoculation. Viral antigen was first visible in cells 4 hr after inoculation. Infected cells contained 20-30 small, brightly fluorescent granules distributed diffusely throughout the cytoplasm. By 6 hr an approximately 3-fold increase in number of granules of antigen had occurred, almost completely filling the cytoplasm with fluorescent material (Fig. 1F). At 8 hr the fluorescent material had begun to form larger aggregates which in some cases appeared to be located near the nuclear membrane but in other cells bore no distinct relationship to the nucleus. Observations at 12 and 22 hr showed no change in this pattern of fluorescence (Fig. 1G).

Chick embryo lung cells inoculated with 1 EID_{50}/cell developed no visible antigen until 8 hr after inoculation. The infected cells at this time contained 15-20 small granules which by 22 hr had increased in number to the point of filling the cytoplasm, resembling the pattern found at 6 hr in cultures inoculated with 100 EID_{50}/cell.

Mumps virus. Mumps virus was studied in 2 cell types. Chicken embryo lung cells were inoculated with the Enders strain (6 EID_{50}/cell) of egg-adapted virus. Since it was not certain that a complete multiplication cycle occurred in these cells, other experiments were performed using human conjunctiva cells and the Dunai strain of mumps virus adapted to serial culture in conjunctiva cells. Conjunctiva cells were inoculated with about one TCD_{50} per cell. With both cell types cultures were harvested and studied at 10, 15, 18, 22, 26, 38, and 65 hr after inoculation. Evolution of the cycle was essentially the same in both cell systems. At 18 hr after inoculation infected cells could first be recognized by the presence of 10-12 small yellow granules scattered throughout the cytoplasm. The nucleus could easily be discerned as a non-fluorescing area. By 26 hr antigen had increased to 5 or 6 large masses that occupied most of the cytoplasm (Fig. 1H). There were no further observable changes in quantity or distribution of antigen and at no time was fluorescence observed within the nucleus.

Discussion. It is clear that in sharp contrast to influenza virus the 3 non-influenza myxoviruses did not at any time in their multiplication cycle produce intranuclear antigen detectable with fluorescein-labeled antibody. The use of antisera from 3 species, employment of several multiplicities of infection, and use of 2 cell types indicate that the observed patterns of antigen development and distribution were not peculiar to a special set of materials or conditions. Ruling out such a possibility was important since in studies now in progress it has been found that under certain circumstances in at least one cell line an incomplete cycle of influenza virus multiplication is not accompanied by intranuclear antigen production although there is abundant cytoplasmic antigen. Additional supporting evidence pointing to lack of intranuclear antigen detectable by fluorescent antibody in cells infected with mumps virus or NDV can be found in several reports. Coons *et al.*(10) located mumps virus in the parotid gland of monkeys using mumps-convalescent monkey serum. Watson studied mumps infection of chicken embryos(11) and of chicken embryo tissues in culture(12) using convalescent monkey serum. Burnstein and Bang(13) identified NDV in infections of the nasal tract of chickens employing NDV-convalescent chicken serum. Wheelock and Tamm(9) made observations on NDV in HeLa cells and Prince and Ginsberg(14) studied the effect of NDV on Ehrlich ascites tumor cells using immune sera produced in rabbits. Most of these studies did not closely follow a single cycle of infection, and adequate levels of antibody against S antigen in their antisera were not confirmed by specific test, but most used antisera that would be expected to contain anti-S antibody and various parts of the multiplication cycle should have been present in their preparations. In all instances, however, antigen was confined to the cytoplasm. In addition, Rountree(15) followed a single cycle of NDV infection in cultured chicken embryo cells using NDV-convalescent chicken serum, and throughout the cycle found antigen only in the cytoplasm.

While the present study indicates that intranuclear production of antigen demonstrable by fluorescent antibody is not characteristic of NDV, mumps, and Sendai viruses, it does not tell us where the S antigen associated with these viruses is produced. It remains possible that the nucleoprotein antigen is produced within the nucleus but in a form that does not combine with antibody under the conditions of the fluorescent antibody technic. On the other hand, it may be that in infections with these viruses S antigen is produced in the cell cytoplasm. Studies are in progress to clarify this point.

The finding that intranuclear antigen production so prominent in the multiplication cycle of influenza and fowl plague viruses is not demonstrable in the cycle of NDV, Sendai, and mumps viruses may add another characteristic to those that seem to separate the myxoviruses into 2 distinct subgroups. The site of antigen production in the cell has not been studied for all the myxoviruses, however, so that evaluation of these observations in this regard must await further information.

Summary. The multiplication cycles of Sendai, mumps, and Newcastle disease viruses were compared with that of influenza A virus in regard to site of antigen production in the cell. Single-cycle infections were followed using fluorescent antibodies prepared from infection-convalescent immune sera shown to contain anti-S and anti-V antibodies. In cells infected with influenza A virus antigen was first detectable in the nucleus and became abundant there by the time antigen appeared in the cytoplasm. In cells infected with Sendai, mumps, or Newcastle disease viruses earliest detectable antigen was found as small granules scattered throughout the cytoplasm. Antigen granules increased in size and number to form large cytoplasmic aggregates and masses. Multiplication of Sendai, mumps, or Newcastle disease viruses did not include production of antigen in the nucleus detectable by fluorescent antibody at any stage of the cycle.

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Antioxidants and Survival Time of Mice Exposed to Multiple Sublethal Doses of X-Irradiation.* (25804)

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It has been suggested that the deleterious effects of ionizing radiation in animals are due, at least in part, to excessive formation of peroxides(1,2). If such were the case, preventing or minimizing peroxide formation following radiation might be expected to have a protective effect. Attempts to counteract radiation injury by administering tocopherol, a naturally occurring antioxidant, however, have been unsuccessful. Furth *et al.*(3) administered alpha-tocopherol in oil to rats prior to irradiation without beneficially improving survival. Haley *et al.*(4) found that intramuscular injections of a water-soluble vitamin E to mice immediately prior to x-irradiation were similarly without protective effect. On the contrary large doses of Vit. E significantly decreased survival following exposure to a lethal dose of total body x-irradiation in mice (4). Since antioxidants may differ in respect to their concentration and distribution in tissues as well as their capacity to inhibit peroxide formation *in vivo,* the present investigation was undertaken to obtain further data on effects of antioxidants of diverse structure and activity on length of survival following exposure to multiple sublethal doses of total body x-irradiation in the mouse.

Procedure. The basal ration employed in the present experiment consisted of cerelose, 59%; casein,[†] 24%; cottonseed oil, 10%; salt mixture,[‡] 5%; cellulose,[§] 2%; and the following vitamins per kg of diet: thiamine hydrochloride, 10 mg; riboflavin, 10 mg; pyridoxine hydrochloride, 10 mg; calcium pantothenate, 60 mg; nicotinic acid, 100 mg; ascorbic acid, 200 mg; biotin, 4 mg; folic acid, 10 mg; para-aminobenzoic acid, 400 mg; inositol, 800 mg; Vit. B_{12}, 150 μg; 2-methyl-1, 4-naphthoquinone, 5 mg; choline chloride, 2 g; Vit. A, 5000 U.S.P. units; Vit. D_2, 500 U.S.P. units; and alpha-tocopherol acetate, 100 mg. The vitamins were added in place of equal amount of cerelose. Male mice of Webster strain were selected at 11 to 14 g in body weight and were divided into comparable groups consisting of 30 animals per group. These were placed in metal cages with raised screen bottoms (5 animals per cage) and fed either basal ration or basal ration plus the supplements listed in Table I. Supplements were added to basal ration in place of an equal amount of cerelose. Food and water were provided *ad libitum.* The animals were fed daily and all food not consumed 24 hours after feeding was discarded. After 6 weeks of feeding, all mice received an exposure of 200 r total body x-irradiation which was repeated once weekly until a total of 1600 r (8 exposures) had been administered. Animals were continued on their respective diets for 126 days after the first x-ray exposure or until death, whichever occurred sooner. All mice that died before the 4th x-ray exposure were not included in tabulation of data. This procedure was employed to minimize variations due to early deaths or atypical responses on the part of individual mice. Data were obtained as to the day after first x-ray exposure when 50% of the animals in each group were dead and average length of survival on each of the diets employed. The following radiation factors were employed: GE Model Maximar 250; 250 kv; 15 ma; 0.5 mm Cu and 1 mm Al filters plus a Cu parabolic filter;[||] HVL, 2.15 mm Cu; target distance to top of box, 82 cm; and dose rate, 15.6 r per

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[†] Vitamin-free Test Casein, General Biochemicals, Chagrin Falls, O.

[‡] Wesson Modification of Osborne-Mendel Salt Mixture, General Biochemicals, Chagrin Falls, O.

[§] Solka Floc, Brown and Co., Boston, Mass.

[||] A nonuniform filter which produces a flat isodose surface of x-ray intensity constructed by the method of Greenfield and Hand(5). Center of filter was 1.7 mm Cu; the edge, 0.5 mm Cu.

TABLE I. Effects of Antioxidants on Survival Time of Mice Exposed to Multiple Sublethal Doses of Total Body X-irradiation.*†

Dietary group	No. of animals	Avg body wt at time of 1st x-irrad. (g)	Day after 1st x-irrad. when 50% of animals were dead	No. surviving‡	Avg length of survival after 1st x-irrad. (days)§
Basal ration	29	29.2	64	2	67.6 ± 5.3‖
Idem + following supplements:					
.25% mixed tocopherols	28	29.4	63	1	65.8 ± 4.6
.25% Santoquin	29	25.4	60	3	66.4 ± 4.5
.25% DPPD	29	28.7	66	5	75.4 ± 5.8
.5% ”	28	28.3	68	6	75.3 ± 6.4
.25% propyl gallate	28	28.7	82	7	83.9 ± 6.8
.5% ” ”	28	28.0	93	9	85.5 ± 7.0
.25% DBH	26	27.4	96	8	89.2 ± 6.7
.5% ”	26	25.5	82	3	84.6 ± 5.5
.25% BHT	24	28.2	95	9	93.5 ± 6.6
.5% ”	28	26.7	>126	16	96.9 ± 7.0

* Each group initially consisted of 30 mice. Animals that died before 4th x-irradiation were not included in Table.

† Test supplements were obtained from following sources: Mixed tocopherols concentrate N.F., Type 4-50 (each g contains 500 mg of mixed tocopherols, equivalent to 372.5 international units of vit. E), Distillation Products Industries, Rochester, N. Y.; Santoquin, Monsanto Chemical Co., St. Louis, Mo.; DPPD, B. F. Goodrich Chemical Co., Cleveland, O.; propyl gallate, Heyden Newport Chemical Co., N. Y.; DBH, Distillation Products Industries, Rochester, N. Y.; and BHT, Nopco Chemical Co., Harrison, N. J.

‡ Experiment terminated 126 days after 1st x-irradiation.

§ Data calculated on basis of avg survival time of 126 days for animals alive at termination of experiment.

‖ Including stand. error of mean calculated as follows: $\sqrt{\dfrac{x^2 - \dfrac{(\Sigma x)^2}{n}}{n-1}} \Big/ \sqrt{n}$, where ‘‘x’’ = length of survival after 1st x-irradiation and ‘‘n’’ is No. of observations.

minute (measured in air, at top of box). The animals to be irradiated were placed in a wooden box divided into 60 equal compartments 1¼″ wide, 3″ long, and 1½″ deep. The partitions and top were made of ⅛″ cellulose acetate sheetings; and top and bottom of each compartment were perforated with holes for purposes of ventilation. The container was rotated slowly on an electrically driven turntable to insure equivalent irradiation.

Results. Findings are summarized in Table I. Significant differences occurred between the various groups in respect to survival following exposure to multiple sublethal doses of total body x-irradiation. Whereas mixed tocopherols and Santoquin (6-ethoxy-2, 2,4-trimethyl-1, 2-dihydroquinoline) at a 0.25% level in the diet¶ and DPPD (N, N′-diphenyl-p-phenylenediamine) at levels of 0.25% or 0.5% of the diet had little if any effect on length of survival, the day 50% mortality was attained or number of animals surviving, propyl gallate, DBH (2,5-di-tert-butyl-hydroquinone) and BHT (butylated hydroxy toluene) at levels of 0.25% or 0.5% of the diet all resulted in increased survival compared to that of animals fed the basal ration. Findings were particularly significant in the case of BHT.

Discussion. Present findings indicate that substances with antioxidant activity can significantly prolong survival following exposure to multiple sublethal doses of total body x-irradiation in the mouse. Not all substances with antioxidant activity, however, were active in this regard. Thus, whereas BHT and to a lesser extent DBH and propyl gallate prolonged survival under conditions of the present experiment, mixed tocopherols and Santoquin were inactive at the levels fed and DPPD exhibited only questionable activity. The protective effect of BHT, DBH and propyl gallate are consistent with the suggestion that the deleterious effects of ionizing radiation are due, at least in part, to peroxide formation(1, 2) and that antioxidants by preventing or minimizing peroxide formation exert a protective effect. The mixed tocopherols, which are naturally occurring antioxidants, were far less active, however, than the synthetic antioxidants BHT, DBH and propyl gallate, suggesting that the latter compounds might be more effective than tocopherols in reaching the site of peroxide action, or in their capacity to inhibit peroxide formation. If such were the case, it is possible that other antioxidants might be even more effective against radiation injury. The possibility has not been excluded, however, that the protective effect of BHT, DBH and propyl gallate is due to some mode of action other than their antioxidant activity.

¶ An additional group was started, fed Santoquin at 0.5% level in the diet. Fifteen of 30 mice died during first 6 weeks of feeding and survivors averaged only 18.1 g in body weight on day of 1st x-irradiation. In view of toxicity of Santoquin at this level of feeding, these animals were discarded and not included in the radiation experiment.

Summary. Experiments were conducted to determine effects of antioxidants on survival time of mice exposed to multiple sublethal doses of total body x-irradiation. Mixed tocopherols and Santoquin at 0.25% level in the diet, and DPPD at levels of 0.25% or 0.5% of diet had little if any protective effect. Propyl gallate, DBH and BHT at levels of 0.25% or 0.5% of the diet increased survival over that on basal unsupplemented ration.

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Effect of Osmotic Diuresis on Renal Blood Flow.* (25805)

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The demonstration that osmotic diuresis has a protective effect against the tubular lesions following renal ischemia, first by Owen and associates(1), and later by Hatcher and his associates(2), arouses interest in what peculiar properties of osmotic diuresis might be responsible for this effect. Very little study has been devoted to effects of osmotic diuresis on renal function and hemodynamics. Earlier studies have been focused on the mechanisms by which osmotic diuresis was produced. This paper presents evidence that osmotic diuresis under certain conditions may affect renal blood flow.

Methods. Patients undergoing differential renal function studies in investigation of hypertension were used in this study. Only patients whose clearance studies revealed renal blood flow and glomerular filtration to be within normal limits were included. After ureteral catheterization and after obtaining a blood and urine blank specimen, priming and maintenance infusions of sodium paraaminohippurate and inulin were begun in accordance with previously described technics(3). Previously, patients had been hydrated by oral intake of at least 1000 cc of water before the experiment began. One hour was allowed for

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TABLE I. Changes in Total Urine Flow, Inulin Clearance, and PAH Clearance after Induction of Osmotic Diuresis. All values are in ml/min. 15 patients.

Before urea infusion			1st period after urea infusion			2nd period after urea infusion		
V	C_{IN}	C_{PAH}	V	C_{IN}	C_{PAH}	V	C_{IN}	C_{PAH}
3.4	121.8	506	6.0	128.9	473	5.4	97.8	452
2.5	107.4	361	4.4	109.5	434	10.7	86.0	460
1.9	50.3	175	6.3	63.2	221	8.2	48.0	180
2.2	80.3	293	5.3	66.4	353	4.8	74.2	364
2.3	60.8	285	7.2	62.7	307	8.8	72.1	256
1.0	105.5	552	3.3	97.2	630	8.5	179.2	751
1.6	94.6	644	3.4	144.6	751			
3.9	41.1	301	8.0	36.9	439	6.0	37.6	349
1.5	79.0	301	1.8	102	409	2.8	107.1	386
1.2	114.9	460	2.5	149.4	554	3.5	146.8	578
.8	83.5	433	2.8	94.0	598	7.5	100.3	552
2.1	101.7	447	3.6	104.9	546	3.8	100.0	492
1.1	114.3	453	2.2	145	553	5.8	117.6	534
.7	55.0	154	4.9	72.8	212	4.8	58.8	168
1.0	107.2	444	1.6	93.6	521	4.2	83.2	453

equilibration following injection of the priming dose; thereafter successive 30 minute clearances were done. By the time equilibration had occurred, the patients complained of mild to severe discomfort secondary to the presence of the catheters and the prolonged immobilization. Despite continued oral hydration, and a decrease in serum osmolality, antidiuresis ensued with pronounced drop in urine output and corresponding rise in urine concentration.

Results. Table I shows changes in clearances of 15 patients following infusion of 4 or 8% urea in saline. Before urea solution was infused, this group of patients without exception had ceased to exhibit water diuresis with a consequent drop in urine flow and increasing urine osmolality. Following infusion of urea, it can be seen that all but one of the patients had a distinct and definite rise in PAH clearance during the first 30 minute clearance period, ranging from 10% to 65% increase. In the second clearance period, the rise in PAH clearances was maintained in the majority of patients.

Changes in inulin clearance were not as striking nor as consistent as for PAH clearance. A large number of these patients actually showed little change or a slight drop in inulin clearance after infusion of urea was begun. Mean rise in inulin clearance following onset of osmotic diuresis was approximately 6.5% during the first clearance period.

In all patients a definite rise in urine output consistent with osmotic diuresis was seen following infusion of urea solution but there were no changes in pulse rate or blood pressure.

Discussion. No conclusions as to the mechanism by which osmotic diuresis may cause a rise in renal blood flow can be made from the present study. Prior to urea infusion all of the patients were in a well-established state of antidiuresis secondary to pain, a stimulus known to depress PAH clearance. It would appear likely that the increase seen in PAH clearance after onset of osmotic diuresis in this situation may be a return to normal flow rather than an actual increase.

Dogs stressed by anesthesia and an operative procedure showed a rise in directly measured renal blood flow after infusion of mannitol (Hostnik *et al.*)(4). The fall in renal flow usually seen after trauma appeared to be lessened.

Osmotic diuresis may benefit renal circulation after stress or trauma.

Summary. A rise in PAH clearance following onset of urea diuresis was observed in 14 patients out of 15 who exhibited antidiuresis secondary to pain following ureteral catheterization.

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Intravenous Histamine and Gastric Pepsin in the Unanesthetized Dog. (25806)

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The effect of histamine on gastric pepsin secretion remains uncertain. Thus, some investigators have concluded that following histamine there is only a "washing-out" of preformed pepsin(1). Others have described a true stimulation similar to that caused by pilocarpine although less potent(2). Still others have mentioned an inhibition of pepsin secretion(3,4). These observations have been made on a variety of animal preparations, including dogs with gastric fistulae or pouches (1), or dogs with vagotomized pouches of the entire stomach(2), or on anesthetized, intubated dogs having intact stomachs(5). To gain more precise information on this subject, we have employed maximal histamine stimulation to the intact canine stomach for a 3-hour period. A quantitative method was used for measuring pepsin.

Methods. Five mongrel dogs (wt. 13-19 kg) were fitted with gastric and duodenal cannulae(6). Gastric content was collected from a rubber tube inserted through the gastric fistula and from a mushroom-type catheter which was pulled against the gastric side of the pylorus and emerged from the duodenal fistula. Histamine diphosphate was infused intravenously at the rate of 1.8 μg of histamine base per kg body weight per minute(7) for 3 hours. Gastric juice was collected every 15 minutes. In the pre-infusion columns of Tables I and II, only those 15 minute samples in which free acid was present were included in "number of samples." Free and total acidity were measured by microtitration(8). Peptic activity was determined by a hemoglobin substrate method(9) and by a turbidimetric method(10). Because the low concentrations of pepsin caused the color value to be present in the unreliable portion of Anson's curve(9), we digested the hemoglobin substrate[†](11,12) with known quantities of crystalline pepsin,[‡] and compared the color value of the unknown gastric juice samples with these. The apparent linear relation between small amounts of a particular sample of crystalline pepsin and color value of the digested filtrate may be seen in Fig. 1. After completion of the animal experiments 2 more samples of crystalline pepsin were obtained from the same company. They gave the same linear relation, but the slopes of the lines were $\hat{Y} = 11X$ and $\hat{Y} = 15X$ rather than $\hat{Y} = 16X$.

Results. Peptic activity as determined by the Riggs and Stadie method (Table II) decreased from the level of the first half-hour of infusion to less than one-third of this activity in the third hour. There were significant differences between velocity constants in the first half-hour and the second half-hour ($p < .01$)

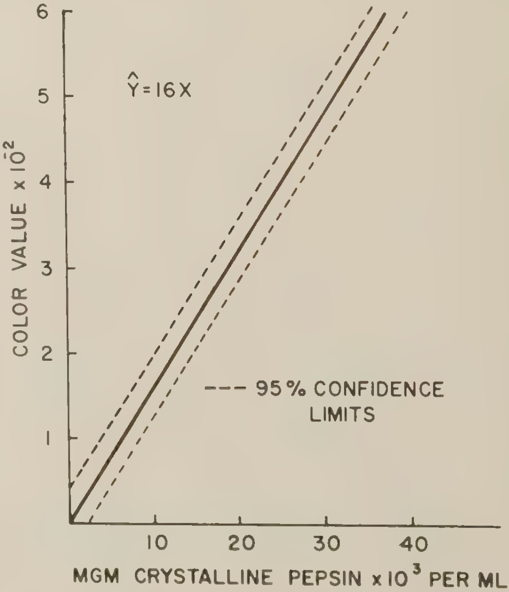

FIG. 1. Relation between pepsin and optical density of digested hemoglobin filtrate.

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* Pre-doctoral Research Fellow, Inst. of Dental Research, U.S.P.H.S., Feb. 1957-58.

[†] Worthington Biochem. Corp.

[‡] Nutritional Biochemicals Corp.

TABLE I. Volume and Acid Response of Gastric Juice in Dogs to Maximal Histamine Stimulation.

	Pre-infusion	First half hr	Second half hr	Second hr	Third hr	Post-infusion
Volume, ml/15 min.	8 ± 5.5* (42)†	31 ± 17.3 (21)	46 ± 12.6 (22)	48 ± 11.4 (44)	40 ± 11.6 (44)	14 ± 8.2 (9)
Free acid conc., meq/l	29 ± 29.4 (31)	85 ± 34.4 (26)	121 ± 20.5 (28)	130 ± 13.2 (56)	131 ± 11.9 (54)	113 ± 17.5 (7)
Total acid conc., meq/l	53 ± 33.5 (25)	109 ± 54.5 (20)	135 ± 30.4 (20)	146 ± 23.2 (40)	141 ± 13.3 (40)	127 ± 12.5 (6)
Free acid output, meq/15 min.	.41 ± .32 (22)	2.62 ± 2.02 (22)	5.76 ± 2.05 (24)	6.19 ± 1.60 (45)	5.16 ± 1.38 (44)	1.77 ± .59 (7)
Total acid output, meq/15 min.	.49 ± .31 (23)	3.42 ± 2.47 (22)	6.77 ± 2.59 (23)	6.95 ± 1.64 (45)	5.59 ± 1.51 (44)	2.01 ± .82 (7)

* Stand. dev. † No. of samples.

TABLE II. Gastric Pepsin Response in Dogs to Maximal Histamine Stimulation.

	Pre-infusion	First half hr	Second half hr	Second hr	Third hr	Post-infusion
Velocity constant ("K" × 10^2)		8.2 ± 3.41 (10)	4.0 ± 1.52 (12)	2.9 ± .93 (24)	2.4 ± .97 (22)	2.0 ± .45 (3)
Pepsin conc. (mg crystalline pepsin × 10^{-2}/ml)	57 ± 50 (9)	21 ± 22 (10)	5 ± 3 (10)	3 ± 4 (20)	3 ± 4 (20)	5 ± 8 (3)
Pepsin output (mg crystalline pepsin × 10^{-2}/15 min.)	131 ± 84 (9)	227 ± 175 (10)	103 ± 51 (10)	53 ± 23 (20)	35 ± 9 (20)	14 ± 8 (3)

and also between those of the second half-hour and the second hour of infusion ($p<.01$). Pepsin concentration, as determined by the quantitative method of Anson, also showed a progressive decrease from pre-infusion levels throughout experiment. Concentration in the first half-hour was noticeably less than pre-infusion concentration ($p<.02$), output of pepsin in the second hour was less than pre-infusion output ($p<.01$), and output in the third hour was less than that in the second hour ($p<.01$).

Summary. Output of hydrochloric acid rose to 5.2-6.2 meq/15 minutes after second hour of a 3 hour intravenous infusion of histamine. Pepsin output, on the other hand, fell from output equivalent to 227 x 10^{-2} mg crystalline pepsin/15 minutes in the first 30 minutes to 35 x 10^{-2} mg crystalline pepsin/15 minutes in the third hour. The high output during the first half-hour supports the "wash-out" hypothesis of Babkin(1), while the continued presence of pepsin after 3 hours could be the result of a "basal" rate of pepsin discharge or a true histamine-stimulated secretion.

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Depression of Serotonin and Norepinephrine Levels in Brain Stem of Rabbit by Ethanol.* (25807)

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Physiologically active amines serotonin and norepinephrine are distributed in the brain in a regular pattern. High concentrations of both neurohormones are found in brain stem and low concentrations are found in cerebrum and cerebellum, and it has been suggested that serotonin is the chemical transmitter of the central parasympathetic system and that norepinephrine is the central sympathetic transmitter(1). The importance of these neurohumoral agents in those regions of the brain where autonomic regulation is believed to occur led us to study the effect of acute and chronic ethanol administration on their concentration in rabbit brain stem. A preliminary report has been made(2).

Materials and methods. White male New Zealand rabbits (4-6 kg) were injected intravenously with ethanol (2 g/kg in normal saline for 5-10 minutes) and then sacrificed at different intervals by intravenous injection of air. The brain was removed as rapidly as possible and either frozen at –5°C or homogenized with 0.1 N HCl. Serotonin was determined by the method of Bogdanski *et al.*(3) and norepinephrine by the method of Shore and Olin(4) using an Aminco-Bowman spectrophotofluorimeter.

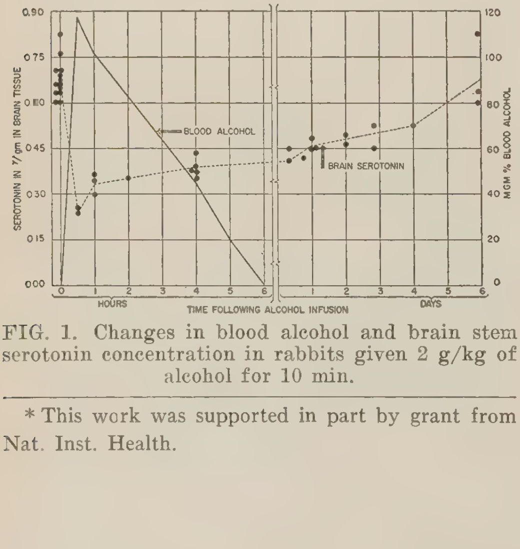

FIG. 1. Changes in blood alcohol and brain stem serotonin concentration in rabbits given 2 g/kg of alcohol for 10 min.

TABLE I. Parallel Changes in Serotonin and Norepinephrine Concentrations* in Rabbit Brain Stem after Ethanol.

	Serotonin		Norepinephrine	
Hr after ethanol	γ/g	% control	γ/g	% control
0	.67 (.60-.82)	100	.46 (.41-.51)	100
1	.33 (.30-.36)	49	.25 (.20-.34)	54
8	.42 (.40-.45)	62	.32 (.30-.34)	69
48	.46 (.45-.48)	69	.38† (.37-.40)	83
72	.49 (.45-.52)	73	.41† (.34-.48)	89
144	.69† (.60-.82)	102	.54† (.51-.56)	117

* Each value represents mean of determinations in 2-10 animals; mean and range are given.

† Avg not significantly different from control value at zero time. All other values different from value at zero time at less than 0.05 level of probability.

Results. Following acute injection of alcohol brain stem serotonin concentration declined rapidly to about 40% of control value at 30 minutes. The level then increased slowly reaching normal value in about 6 days (Fig. 1). Norepinephrine concentrations were reduced to about 55% of control values (0.46 μg/g) after 1 hour, then rose *pari passu* with serotonin concentrations to 100% of normal in 4-5 days (Table I). Shape and duration of both curves were very similar to patterns produced by intravenous injection of reserpine (5,6). Daily administration of ethanol (2 g/kg) intravenously to 4 rabbits for 7 days resulted in a significant depression of serotonin from a control average of 0.67 μg/g (range 0.60-0.82) to 0.32 μg/g (range 0.30-0.35) and a significant depression of norepinephrine from a control average of 0.46 μg/g (range 0.41-0.51) to 0.24 μg/g (range 0.20-0.26).

Discussion. This depression of brain stem levels of serotonin and norepinephrine by ethanol strongly resembles the depression produced by reserpine in that depletion persists long after the drug has disappeared. Blood alcohol levels were too low to measure 6 hours following acute dose, whereas depression of

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brain stem serotonin lasted 15-20 times as long. This action of ethanol furthermore does not appear the result of hypnotic effect of the drug since common hypnotic and analgesic agents such as phenobarbital, barbital, morphine, and scopolamine do not lower brain stem serotonin levels in the rabbit(7). It has also been shown that duration of hypnosis in mice injected intraperitoneally with 4 g/kg of ethanol is potentiated by reserpine(8). Our observations that ethanol also produces an effect on the 2 neurohormones closely paralleling that of reserpine seem to provide the most reasonable interpretation for this potentiation.

A plausible hypothesis of neurohumoral action and the effect of reserpine upon these phenomena has been advanced by Brodie, Spector, and Shore(9). These authors postulate that binding sites for serotonin and norepinephrine exist in the brain stem, which provide a mechanism for controlled release of serotonin and norepinephrine in facilitating conduction. These authors furthermore attribute the sedative, hypotensive and other effects of reserpine to impairment of binding sites of serotonin and norepinephrine, which releases stored amines quickly and renders binding sites incapable of taking up newly synthesized amines. According to their hypothesis, these neurohormones are then liberated directly from a synthetic locus in the cell at a rate which may produce a physiologic effect if concentration at receptor site reaches a sufficiently high level. Under these conditions, rate of accumulation of amines will be dependent upon its rate of formation and its rate of inactivation by monoamine oxidase. For example, a slowly-formed amine may be metabolized as fast as it is formed while a more rapidly-formed amine may accumulate at receptor sites in concentrations sufficiently high to produce a response. It is known that brain serotonin is synthesized far more rapidly than brain norepinephrine and Brodie *et al.*(9) concluded that sedative effects of reserpine could be explained most readily by persistent bombardment of central receptor sites by free serotonin. The parallel depletion of both neurohormones and duration of the effect produced by ethanol are so similar to that produced by reserpine, that it is tempting to invoke the same mechanism to account for the hypnotic and sedative effects of the 2 drugs. This concept, if verified, would provide a physiological basis for the well-known mood depression of chronic alcoholics.

Summary. Evidence is presented which indicates that intravenous infusion of 2 g/kg of ethanol produced depression of both serotonin and norepinephrine levels in the brain stem of rabbit. The parallel depletion of both neurohormones persisted long after ethanol had disappeared, an effect which is reminiscent of the action of reserpine. Chronic administration of alcohol for 7 days produced 50% decrease in both serotonin and norepinephrine levels in rabbit brain stem.

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Ethanol-1-C^{14} and Acetate-1-C^{14} Incorporation into Lipid Fractions in the Mouse. (25808)

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There is general agreement that the metabolism of ethyl alcohol proceeds through acetaldehyde to enter the acetate pool. From this point there is participation in all reactions involving the remaining 2-carbon fragment, such as fatty acid and cholesterol synthesis and oxidation *via* the tri-carboxylic acid cycle. Schulman *et al.*(1) have found that the protein, glycogen, fatty acids and cholesterol from rats given a single dose of radioactive alcohol contained 2 to 3 times the labelled carbon in these same constituents from rats receiving C^{14} labelled acetate. When report appeared, we had begun similar experiments though of somewhat different design. Since our results differed somewhat from those of Schulman, we considered it worthwhile to pursue our studies further. We found that tissue fatty acids, non-saponifiable fraction, and phospholipids take up almost equal amounts of C^{14} after either radioactive alcohol or acetate administration.

Methods. In one set of experiments, pairs of male C-57 B mice were matched in body weight and placed in separate cages where drinking water contained approximately one μc/ml of ethanol-1-C^{14} or of acetate-1-C^{14}. When solutions prepared from commercially available ethanol-1-C^{14} and acetate-1-C^{14} were not of equal activity, as determined by Packard Tri-Carb scintillation counter, appropriate dilutions were made until the same activity level was measured in each. Non-radioactive acetate or ethanol was added so that final concentration of additives was 0.2 M. The animals could not tolerate a higher concentration of acetate without loss of weight. To ethanol-water solution was also added 0.2 M NaCl to match the sodium concentration in the acetate solution as closely as possible. Mice remained on this regime with *ad lib.* feeding of Purina Rat Chow for 5 days, then, 12 hours before autopsy, the remaining water containing radioactive constituents was measured and replaced by fresh water. This time delay before autopsy was chosen to allow complete incorporation of imbibed alcohol and acetate into the tissues. When different amounts of water were drunk by the 2 mice, appropriate correction factor was applied to final result. In other experiments, radioactive solutions prepared in same manner as drinking water were injected in amounts of one ml, 3 times daily. These mice were provided with fresh drinking water and laboratory chow *ad lib.* Injections were carried on for 5 days, the last injection occurring 12 hours before autopsy. At autopsy, the gut, liver, brain, and a piece of genital and kidney fat were removed from each animal and each homogenized separately in a 2:1 chloroform-methanol mixture (1 g tissue/20 ml solvent). Homogenized mixtures were allowed to stand overnight, then centrifuged to remove cellular debris. After evaporation of supernatant solution, the dried material was again taken up in chloroform-methanol, centrifuged again, and evaporated to dryness. The residue was dissolved in 3 ml of petroleum ether and phospholipids were precipitated according to the method of Sinclair and Dolan(2). After overnight in refrigerator, the supernatant was decanted and saved for further treatment, while the precipitate was redissolved in petroleum ether and again reprecipitated. The precipitate was again dissolved, plated on tared planchets, and specific activity was determined. The supernatant from the first precipitate was evaporated to a small volume and saponified in 30% alcoholic potassium hydroxide. The saponified mixture was extracted with petroleum ether, and the extracts pooled, evaporated to dryness, redissolved in petroleum ether, and plated as the non-saponifiable fraction. To the residue after extraction, was added a small amount of water and the mixture was acidi-

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* Deceased Sept. 19, 1959.

TABLE I. Specific Activity of Lipid Fractions from Various Organs from a Pair of Mice Ingesting Ethanol-1-C^{14} or Acetate-1-C^{14} in Drinking Water for 5 Days.

	Ethanol-1-C^{14}	Acetate-1-C^{14}
	cpm/mg	
Phospholipids		
Liver	1136	1003
Gut	1183	1048
Brain	450	474
Non-saponifiable fraction		
Liver	1041	1462
Gut	1031	861
Brain	117	93
Fatty acids		
Liver	1296	1531
Gut	719	618
Brain	253	245
Fat	154	201

fied. Precipitated fatty acids were collected on filter paper, washed, dried, dissolved in acetone, and plated. All plating was done on aluminum or copper planchets, and radioactivity was measured in Nuclear-Chicago gas flow counter, Model 183B. Appropriate correction for self absorption was made by means of previously determined curve. In all, 7 pairs of animals were used, 5 pairs receiving the radioactive compounds in drinking water, and 2 pairs by injection.

Results. The labelling pattern of a typical experiment is shown in Table I. As may be expected, liver and gut showed a high degree of labelling in fractions isolated, while brain and fat fractions were not as active. In general, there was no difference in ratio of carbon uptake from these 2 compounds whether radioactive agents were administered by mouth or by injection. A difference in absolute counts/minute/mg was observed, however, with fractions in injected animals showing greater specific activity. A great range in values of ethanol/acetate uptake ratios was observed as might be expected in this type of experiment. The extremes varied from 0.32 to 6.28. When ratios in individual tissues were averaged, however, values approaching unity were obtained with one exception (Table II). The non-saponifiable fraction from the gut showed ethanol/acetate uptake ratios of greatest variation, the average value being nearly 3. In all other cases, ethanol/acetate uptake ratios fell closer to 1 than to values of 2-3 obtained by Schulman *et al.*(1). Dissimilar experimental plans such as species differences (rat *vs.* mice) and the effect of one injection *vs.* chronic administration may account for the difference in results.

Discussion. Bernhard(3) was the first to show that ethanol is an effective acetylating agent and therefore enters the acetate pool in the organism. Bloch and Rittenberg(4) further demonstrated that deutero-ethanol, when administered in diet of rats, gives rise to deutero-cholesterol. Since that time, there has been little work on the nature of entry of ethanol into the acetate pool, although the definition of "active acetate" has changed a great deal during the intervening period. At present, there is incomplete knowledge of the specific enzyme by which acetaldehyde is oxidized to acetate, although most recent evidence points to the diphosphopyridine nucleotide enzyme, aldehyde dehydrogenase as the chief agent(5).

If ethanol and acetate are to label equally their metabolic products, it is necessary that they enter the metabolic pool at approximately equal rates, as seems to be the case here. It is also necessary that there be no major accessory metabolic pathways which selectively exclude either compound, *e.g.*, a branching pathway at the acetaldehyde stage of ethanol oxidation to yield such compounds as acetoin or 5-carbon sugars. Since tagged alcohol and acetate in our experiments seemed equally able to label various lipid compounds,

TABLE II. Average Ratios of Specific Activities of Lipid Fractions from Pairs of Mice to Which Ethanol-1-C^{14} or Acetate-1-C^{14} Had Been Administered. Figures indicate avg of 7 ratios of ethanol-mouse fractions/acetate-mouse fractions, while figures in parentheses denote range.

	Phospholipid	Non-saponifiable fraction	Fatty acids
		Eth/Ac	
Liver	1.39 (1.03-1.91)	1.34 (.71-2.33)	1.11 (.66-1.94)
Gut	1.13 (.86-1.59)	2.97 (.78-6.28)	1.18 (.43-1.94)
Brain	.94 (.64-1.31)	1.14 (.71-2.10)	.76 (.53-1.03)
Fat			1.15 (.32-2.00)

accessory and selective pathways, if existing at all, would seem to be of minor significance in relation to the amount metabolized along the conventional pathways.

Summary. Pairs of mice were administered either by mouth or by injection equal amounts of ethanol-1-C^{14} or acetate-1-C^{14}. The phospholipid, non-saponifiable fraction, and fatty acid fractions of liver, gut, brain, and fat were isolated and specific activities of these fractions were compared. Ethanol-1-C^{14} and acetate-1-C^{14} contributed approximately equal labelling to tissues investigated.

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Inhibition of Neutrophil Mobilization by Colchicine.* (25809)

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Colchicine is a powerful mitotic poison reported to cause extensive bone marrow damage, arresting division of both developing leukocytes and immature erythrocytes(1). In contrast to the depressive effects upon bone marrow, colchicine injection provokes a short-lived leukopenia followed by a marked increase in numbers of circulating granulocytes in the blood of dogs, rabbits, and mice(2,3). The relationship between bone marrow inhibition and peripheral blood leukocytosis is a puzzling one and remains to be elucidated. Therefore, it was felt that it would be of interest to investigate another parameter of this problem, namely ability of the animal to mobilize neutrophils following colchicine treatment.

Methods. Male rats of the Sprague-Dawley strain and weighing 200-240 g were used. All experimental rats received a single, subcutaneous injection of 0.2 mg colchicine (Lot 920 LPA, S. B. Penick Co.) dissolved in 0.2 ml of 0.9% saline; control animals received 0.2 ml of 0.9% saline. Immediately following subcutaneous injection and at 24, 48, 72 and 96 hours thereafter, groups of 6 animals were challenged by an intraperitoneal injection of 1 μg Piromen,† a Pseudomonas polysaccharide complex (Baxter Lab.) dissolved in 10 ml 0.9% sterile, nonpyrogenic saline. These injections were administered to animals while they were under light ether anesthesia. The rats were sacrificed 5 hours after intraperitoneal injection of bacterial endotoxin. Leukocytes in the peritoneal fluid were harvested and their numbers and types determined from hemacytometer counts and from Wright-Giemsa-stained smears. Previous work(4,5) had established the efficacy of this treatment in evoking a marked neutrophilia in peritoneal fluid of normal, untreated rats.

Results. Fig. 1 summarizes the effects of colchicine upon the pattern of neutrophil mobilization at various times following its injection. Rats challenged by an intraperitoneal injection immediately following injection of colchicine revealed a drastic reduction in numbers of neutrophils harvested from the peritoneal fluid 5 hours later. Such fluid contained less than 8 million neutrophils in contrast to over 59 million neutrophils that were obtained from non-colchicinized, control animals. Twenty-four hours after colchicine injection, challenged rats mobilized neutrophils

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* Supported by grant from USPHS.

† We are indebted to Dr. L. G. Ginger for Piromen used.

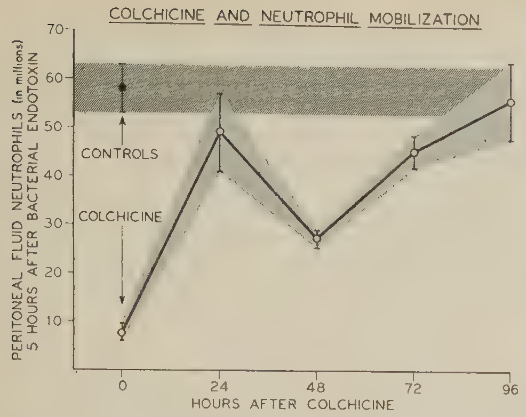

FIG. 1. Effects of a single inj. of colchicine upon neutrophil mobilization into peritoneal fluid of rats induced by inj. of bacterial endotoxin. Each circle represents mean value for 6 rats. Vertical lines represent ± 1 stand. error of mean.

in a manner comparable to the controls, but at 48 hours fewer cells than normal were mobilized. This was still somewhat the case at 72 hours. However, the normal pattern of mobilization was eventually restored since animals challenged 96 hours after colchicine treatment responded by massing normal numbers of neutrophils into the peritoneal fluid. Morphologically, the neutrophils in all the groups tested appeared to be of the mature variety, resembling those seen in circulating blood of untreated rats. Peripheral white blood cell counts revealed a transient leukopenia which was followed by a marked increase in circulating neutrophilic granulocytes.

Discussion. The most striking inhibition of neutrophil mobilization occurred in animals challenged immediately after injection of colchicine. It is unlikely that the anti-mitotic effects of colchicine played a crucial role in this phenomenon in view of the tremendous reserve of neutrophils possessed by the rat (6) and the rapidity with which this inhibition developed. Furthermore, the depression in numbers of mobilized neutrophils was seen at a time when similarly-treated animals have been reported to display a peripheral leukocytosis(2,3). There is evidence that colchicine can exert a variety of actions that apparently are not related to its effects upon mitosis. For example, colchicine has been shown to alter the morphology of the mast cell(7) as well as having effects on the nervous system, muscle and vascular system(1). Of direct interest to the problem in hand, are the findings that epinephrine(8), large doses of bacterial endotoxins(9) and hemorrhagic shock(10) also have the ability to inhibit diapedesis of neutrophils. It is possible that all of the aforementioned treatments as well as the effects noted following colchicine may act *via* a common pathway which affects the vascular system.

A second reduction in mobilization of neutrophils was observed in rats that had been challenged by endotoxin 48 hours after being injected with colchicine, and this response did not revert to normal until 96 hours after injection. This secondary loss of ability on the part of the animal to mobilize neutrophils may be a reflection of the well-known anti-mitotic and destructive actions of colchicine upon bone marrow.

The importance of neutrophil mobilization as a defense mechanism during the first few hours following bacterial invasion has been described(8). It is felt that the use of bacterial endotoxins to stimulate an influx of neutrophils, as in the present experiments, mimics, in many ways, the response to an actual infection. Thus, the possibility must be considered that, under certain conditions, colchicine treatment may render the organism more susceptible to bacterial infection. This depressive effect of colchicine upon neutrophil mobilization is of more than academic interest, since this drug is widely used in treatment of gout while its mode of action in this disease is still a mystery(11).

Summary. A single injection of colchicine markedly inhibited ability of rats to mobilize neutrophils. The greatest inhibition was seen when animals were challenged immediately following colchicine injection. A secondary inability to mobilize neutrophils appeared at 48 hours and was still present at 72 hours following colchicine treatment. The significance of these phenomena is considered.

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Response of Mouse Breast Tumors to L-Triiodothyronine and Irradiation.

(25810)

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In 1951 it was shown that mice made hyperthyroid by oral administration of thyroxine showed increased mortality when given total body irradiation(1). A year later L-triiodothyronine (T3) was isolated from human plasma(2). T3 by various assays was several times as active as thyroxine, and it was postulated that thyroxine is deiodinated to T3 in peripheral tissues before exerting its effect(3). In animals peak physiologic activity with increased oxygen consumption was exhibited by fourth day of T3 administration(4). L-triiodothyronine was strongly accumulated by Ehrlich mouse ascites carcinoma cells(5). Increased glycolysis without increased mitotic rate of growing tumor cells in tissue culture was observed after treatment with T3(6). Stein and Griem studied the effect of combined T3 and x-ray therapy on myeloid chloroleukemic rat tumor, mouse neuroblastoma, and human bronchogenic carcinoma(7). They noted more marked tumor regression after combined therapy so that a lower x-ray dose would accomplish the same therapeutic result. The skin reaction was accelerated. The purpose of this study was to evaluate the effect of x-radiation on transplanted mouse breast adenocarcinoma when animals had been made hyperthyroid by administration of L-triiodothyronine.

Method. Randomly selected ZxC57 black F_1 male and female hybrid mice, aged 3 to 6 months, were kept in cages in air-conditioned room maintained at 72°F. An ample supply of tap water and Purina fox chow was always available. Twenty-five mg of T3 were dissolved in 25 cc of propylene glycol and 25 cc of sterile water and kept in freezing compartment. The same material was used throughout preliminary toxicity studies as well as actual experiment. Five groups of 5 mice each were injected intraperitoneally with .25, .50, .75, 1, and 2 mg/kg respectively, for 4 consecutive days. Weights were recorded daily and animals observed for signs of hyperthyroidism. The first 3 groups developed moderate diarrhea and a transient weight loss, most marked on third day. Weights were back to normal by 6th day. In groups receiving 1 mg/kg weight fluctuation was less marked but signs of hyperthyroidism were evident. The animals had diarrhea, loss of coat sheen, marked increase in activity, increased water and food consumption without increase in weight. The group receiving 2 mg/kg showed polydipsia, transient weight loss, bulimia, increased respiratory rate, and loss of coat sheen. By 5th day these animals showed extreme lassitude. Dose of 1 mg/kg was optimum and this was in the dose range employed by Stein and Griem(7). Spontaneous adenocarcinoma of the breast originating in a Z female mouse and transferred by serial transplantation for 2 generations was processed

TABLE I. Animal Survival.

Group	T3 (mg/kg)	X-ray (r)	No. of animals	Avg survival (days)*
1	1	0	25	19.4 ± 1.6[1]
2	0	4500	"	42.2 ± 3.2[1,2,3]
3	1	4500	"	47.1 ± 3.2[1,2]
4	0	0	15	28.8 ± 2.2

* Includes stand. error.

[1] Highly significant difference from Group 4 ($P = .001$).

[2] Highly significant difference from Group 1 ($P = .001$).

[3] Insignificant difference from Group 3 ($P = .28$).

with a tissue press. A suspension of fresh tumor cells in sterile physiologic saline was injected subcutaneously in ventral aspect of hind leg of 90 ZxC57 black F_1 hybrid mice. At end of 25 days the tumors averaged 11 mm in diameter and the animals were randomly divided into 4 separate groups. The first group of 15 animals received no therapy and was maintained as control. Two groups of 25 animals were treated with T3, 1 mg/kg intraperitoneally, for 4 days and on the morning of fifth day one of the T3 treated groups as well as a control group of 25 animals were treated with 4500 r in air (140 KV, HVL 3 mm Al, FSD 13 cm, dose rate 200 r/min). Radiation was delivered to the tumor-bearing leg while the rest of body was shielded with lead (Table I).

Results. The following parameters were observed: Life span, tumor size, and body weights, presented in Fig. 1, 2 and 3.

A statistically significant increase in life span was noted in groups receiving x-ray therapy as compared to controls. There was also a significant decrease in longevity of the T3 control group compared to the group which received no therapy. No significant difference in life span noted between the group receiving T3 in addition to x-ray treatment compared to group receiving x-ray alone (Fig. 1, Table I).

Tumor growth rates were studied by measuring breadth and length of tumor with a Vernier caliper. These values were multiplied and the products averaged for graphing. In group receiving T3, tumor growth rate was accelerated to a significant degree compared to controls which received no therapy. In

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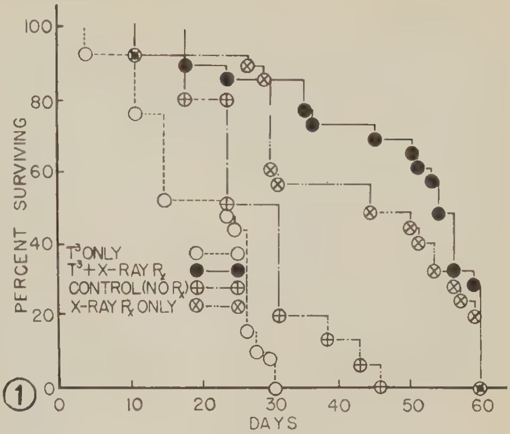

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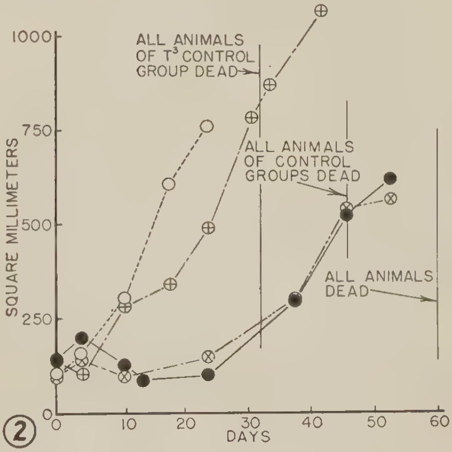

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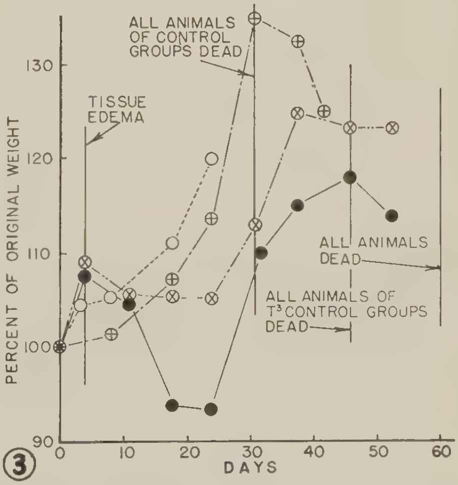

FIG. 1. Life span.

FIG. 2. Tumor size. On day 24 a significant difference between tumor size of control groups was demonstrated ($P = .001$).

FIG. 3. Animal wt.

groups receiving x-ray treatment an initial increase is tumor size, attributed to tissue edema, was noted. This was followed by regression and ultimate tumor regrowth. At time of death all animals in each group exhibited huge tumors. These tumors contributed to approximately one-third of animals' total body mass (Fig. 2).

Control groups gained weight more rapidly than the group receiving x-ray treatment. In groups receiving irradiation, initial slight weight gain might be attributed to tissue edema. This was followed by weight loss which paralleled tumor regression. Weight gain accompanied tumor growth in all groups but several days prior to death weight loss was observed. The group receiving T3 plus x-ray averaged 5 g less in weight than the other groups (Fig. 3).

Two weeks following irradiation maximum tumor regression and skin reaction were noted. In the group receiving T3 plus x-ray, moist epidermitis and superficial ulceration were observed, while in the group which received x-ray alone epilation and a brisk erythema were most commonly seen. In several animals receiving both T3 and x-ray limb amputation occurred subsequent to irradiation.

Conclusions. 1. Growth rate of transplanted mammary tumor in ZxC57 black F_1 hybrid mice treated with L-triiodothyronine was increased. 2. Average life span of tumor-bearing mice receiving L-triiodothyronine was significantly shortened. 3. In mice receiving L-triiodothyronine and x-ray, increased skin reaction was noted. 4. There was no significant difference between average life span of tumor-bearing groups receiving combined irradiation plus triiodothyronine and the group which received local irradiation only.

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Effect of Single Dose of DL-Serine on Lipide Phosphorylation in Kidney of Rats.* (25811)

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Interference of plasma lipide metabolism in degenerative kidney lesions has been reported (1). The present study is, to our knowledge, the first to investigate metabolism and distribution by column chromatography of phospholipides in experimental renal necrosis produced by administration of a single dose of DL-Serine.

Methods. Experimental necrosis was produced by administration of a single dose of DL-Serine by the method of Fishman and Artom(2). Male albino rats of Sprague-Dawley strain weighing 80-100 g were fed 10% casein diet(3) for 10 days supplemented daily with a B vitamin solution(4) not containing pyridoxine. Control animals received a similar diet daily supplemented with Vit. B solution containing pyridoxine. After being on the diet for 10 days, the rats were stomach-tubed with single dose of 100 mg of DL-Serine; control animals received water. Forty-

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TABLE I. Effect of Single Dose of DL-Serine on Lipide Phosphorylation in Kidney of Rats.

		Phospholipide phosphorus	
	No. of rats	mg/g wet wt	Relative specific activity
Control	20	.74 ± .15	.094 ± .009
Necrosis	27	.59 ± .12*	.309 ± .079*

Test of significance was applied to difference between mean of control and experimental values. Probability for chance occurrence of this difference was * <0.01.

eight hours later the rats were killed by decapitation. All animals were injected intraperitoneally with 1 ml of physiological saline containing 4 μc of P^{32} as NaH_2PO_4 6 hours before sacrifice. Kidneys were removed, rapidly weighed, a small sample taken for histological examination, and the remainder divided into 2 fractions. From one fraction, acid soluble phosphorus was removed by extracting with 10% TCA containing 0.4 M $MgCl_2$(5), and radioactivity and total P(6) were determined. The other fraction was covered with alcohol, and lipides extracted with alcohol-ether and purified with chloroform(7). Radioactivity(7) and phosphorus(6) were determined on aliquots of the chloroform solution. As a measure of phospholipide turnover, relative specific activities(8) were calculated. Additional groups of rats (240-400 g) were fed similar diet for 18 days, then they received, by stomach-tube, either a single dose of 100 mg of DL-Serine (experimental) or water (controls). Thirty hours later animals were killed by decapitation. Six hours before sacrificing 20 μc of P^{32} was administered intraperitoneally. Kidneys were removed, rapidly weighed and a small section taken for histological examination. Kidneys were pooled from 4 rats for each experiment except for one control group which contained 2 rats. The kidneys were homogenized in the cold for 2 minutes in 95% ethanol in Potter-Elvehjem homogenizer then allowed to stand in the dark at room temperature 4 hours. Lipides were extracted, chromatographed on silicic acid columns and eluted with chloroform-methanol by the method of Hanahan(9) using a Gilson Medical Electronics fraction collector. Radioactivity(7) and phosphorus(6) were determined on aliquots of the chloroform-methanol fractions. Data are reported as means ± standard dev.

Results. Histological examination of all kidneys showed marked degenerative lesions as reported by Morehead *et al.*(10). The effect of a single dose of DL-Serine on lipide phosphorylation in kidney of rats is shown in Tables I and II. To evaluate the significance of results, the *t* test of significance(11) was applied to the difference between mean of controls receiving saline and experimental values. The data in Table I show that DL-Serine necrosis produces a statistically significant decrease in phospholipide content. There is a statistically significant increase in lipide phosphorylation in the necrotic kidneys. Phospholipides of the kidney were separated by column chromatography to elucidate which class of phospholipides is involved in production of the necrosis. Hanahan's(9) method of column chromatographic separation of the classes of phospholipides fractionates them into: lecithins, phosphoinositides and sphingomyelins. However, by this method phospha-

TABLE II. Kidney Phospholipide Composition and Specific Activities Following Administration of Single Dose of DL-Serine.

			% total lipide phosphorus			
	No. of exp.*	mg/g wet wt	Phosphatidylethanolamine-serine	Phosphatidylinositol	Lecithin	Sphingomyelin
Control	4 (14)	.68 ± .05	34.9 ± 3.0	12.9 ± 2.7	46.8 ± 4.4	3.1 ± 1.9
Necrosis	3 (12)	.33 ± .20‡	57.6 ± 4.5†	22.2 ± 5.0†	18.4 ± 6.1†	2.2 ± .2
			Specific activities of lipide phosphorus			
Control	4 (14)	18.9 ± 2.0	4.0 ± 1.5	7.3 ± 1.6	8.7 ± 2.1	5.1 ± 2.3
Necrosis	3 (12)	28.5 ± 4.9 †	10.0 ± 4.8‡	12.9 ± 6.4	11.0 ± 1.1	11.6 ± 3.0†

* Values in parentheses are total No. of rats/series.

Test of significance was applied to difference between mean of control and experimental values. Probability for chance occurrence of this difference was † <0.01; ‡ <0.05.

tidyl serine is mixed with phosphatidyl-ethanolamine. There is a significant increase in % of total lipide phosphorus in the necrotic kidney in phosphatidylethanolamine-serine and phosphatidylinositol fractions (Table II). However, a decrease occurs in the lecithin fraction. The increase in lipide phosphorylation in the necrotic kidney apparently involves only phosphatidylethanolamine-serine and sphingomyelin fractions. These results demonstrate that phospholipides of the kidney are involved in production of kidney necrosis from administration of a single dose of DL-Serine. These phospholipide changes may be a reflection of the degenerative process since these lipides make up 16-32% dry weight of the cellular mitochondria, microsomes and nuclei (12).

Summary. 1) The effects of a single dose of DL-Serine on lipide phosphorylation in kidney were studied in the rat. 2) A statistically significant decrease occurred in phospholipide phosphorus of the necrotic kidney as compared to controls. However, there was a 3-fold increase in lipide phosphorylation as shown in the relative specific activities values. 3) A statistically significant increase occurred in % of total lipide phosphorus in phosphatidylethanolamine-serine and phosphatidylinositol fraction, in the necrotic kidney as compared to controls. However, a decrease occurred in the lecithin fraction. This increase in lipide phosphorylation in the necrotic kidney apparently involved only the phosphatidylethanolamine-serine and sphingomyelin fractions.

The authors express thanks to Dr. Theodore Snook for histological examination and to Cathrine Snyder for assistance in these experiments.

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Isolation of α- and β-Melanocyte Stimulating Hormones (MSH) on a Preparative Scale.* (25812)

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The need for large amounts of α- and β-MSH for investigation of their physiological significance in mammals and particularly for the study of effects of β-MSH on the central nervous system(1) and for further studies on biosynthesis of ACTH from α-MSH(2), prompted us to develop still other methods for isolation‡ of these 2 substances on a preparative scale.

Methods and materials. Starting materials for purification were an MSH concentrate

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‡ For review on isolation and structure of α- and β-MSH, see Harris(3).

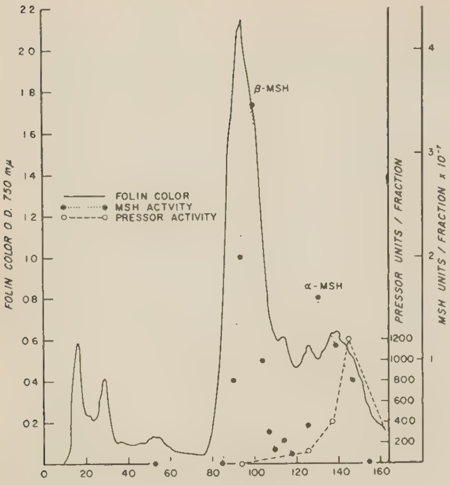
(Armour and Co.) of porcine origin(4) with potency of 3-5 x 10^5 MSH U/mg and Pitressin Intermediate(5,6), 1.5 x 10^5 MSH U/mg (Parke, Davis & Co.). Reagent grade diethylaminoethyl cellulose (DEAE) anion exchange adsorbent (0.88 m equiv/g) purchased from Brown & Co., was treated before chromatography(7). Other details of chromatography, including operation of columns and methods for following peptide concentration have been described(5,6,8). Bioassay for MSH activity is the *in vitro* assay of Shizume *et al.* (11) with minor modifications, using some of their original MSH Standard. As utilized here the assay is highly reproducible as exemplified by standard errors for composite slope b and index of precision λ, calculated(12) for 22 assays in triplicate or quadruplicate ($b_c = 1.41 \pm 0.14$; $\lambda_c = 0.247 \pm 0.027$). Other bioassays as described in(6).

Results. The first step in purification of α- and β-MSH is chromatography on CMC as in the method of Steelman and Andersen(4), but using different conditions. Fig. 1 shows chromatographic pattern and biological activities obtained on column 2.8 x 52 cm with hold-up volume of 160 ml. MSH concentrate (5 g) was applied on column equilibrated with 0.005 M, pH 4.6 ammonium acetate. Gradient to 0.1 M, pH 7, buffer through 1000 ml mixing flask was started at tube No. 29, and 20 ml fractions were collected. Fractions 91-102 yielded after lyophylization 1.7 g β-MSH with specific activity of 0.8 x 10^6 U/mg. Approximately 300 mg of α-MSH similarly obtained from fractions 130-154 had a potency of 2 x 10^6 MSH U/mg and pressor activity of 50 U/mg.

β-MSH could be further purified on DEAE cellulose (Fig. 2). The material (1.7 g) dissolved in 55 ml glass distilled water was adjusted to pH 7 with NH_4OH before chromatography. The column 1.6 x 87 cm contained 31 g DEAE equilibrated with 0.0075 M, pH 7 ammonium acetate, with hold-up volume of 120 ml. The gradient to 0.175 M, pH 7 buffer through 1000 ml flask was started after 2 hold-up volumes and 10 ml fractions were collected. Lyophylization of fractions 54-64 yielded 200 mg of β-MSH with specific potency of 4-5 x 10^6 U/mg. Overall recovery of β-MSH was slightly higher than 20% of total MSH activity present in concentrate. It was pointed out by Lerner and Lee(9) that α-MSH is the main MSH component of hog pituitary gland. β-MSH was electrophoretically homogeneous and relatively stable on storage *in vacuo*.

α-MSH could be further fractionated by zone electrophoresis in pyridine acetate buffer in a large vertical apparatus(10). α-MSH (565 mg) was introduced on column of ethanolyzed cellulose 4 x 160 cm, containing 0.1 M, pH 4.9 pyridine acetate as an electrolyte. After electrophoresis at 2300 volts (200-250 mA) for 50 hr at 3°C, the column (1600 ml) was displaced and collected automatically in 10 ml fractions. α-MSH emerged in fractions 49-62 and was freed from components with a lower cathodic mobility and from the bulk of vasopressin moving ahead of α-MSH. After lyophylization 73 mg of α-MSH with a potency up to 1 x 10^7 MSH U/mg were obtained. Pressor contamination of this material was 3-4 U/mg.

It is difficult to separate α-MSH and lysine vasopressin on CMC on analytical or preparative scales with a gradient of 0.02 M, pH 4.6 to 0.1 M, pH 7 ammonium acetate, as α-MSH emerges on the descending slope of vasopressin (6). A somewhat better separation can be achieved using different conditions for chromatography: Precipitate B (a concentrate of posterior pituitary peptides(6) from Pitressin Intermediate) was chromatographed routinely in 5 g amounts on CMC; the basic peptide fraction (0.6-0.7 g) contains as its main components vasopressin and α-MSH, emerging as a single peak. Chromatography of 40 mg of this α-MSH-vasopressin peak on CMC on an analytical scale is shown in Fig. 3. The column, 1 x 62 cm was equilibrated with 0.03 M, pH 5.7 ammonium acetate. A gradient to pH 7, 0.1 M buffer through 250 ml flask was applied after 2 hold-up volumes and 2 ml fractions were collected. Under these conditions the relative order of elution is reversed, α-MSH emerging first, followed by lysine-vasopressin. These peptides however are still contaminated with each other (Fig. 3).



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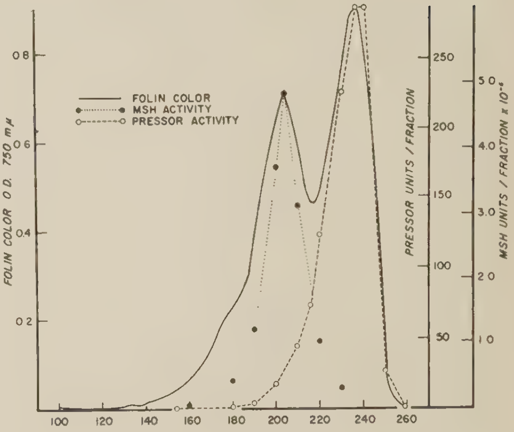

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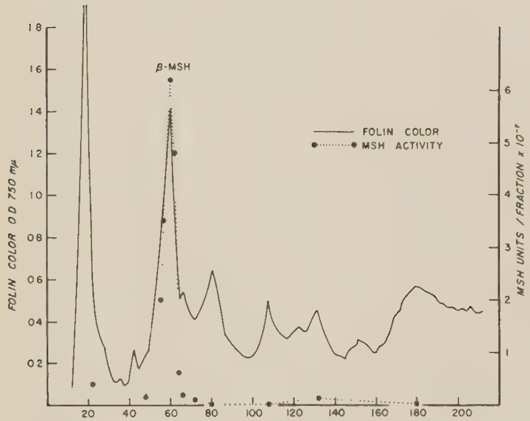

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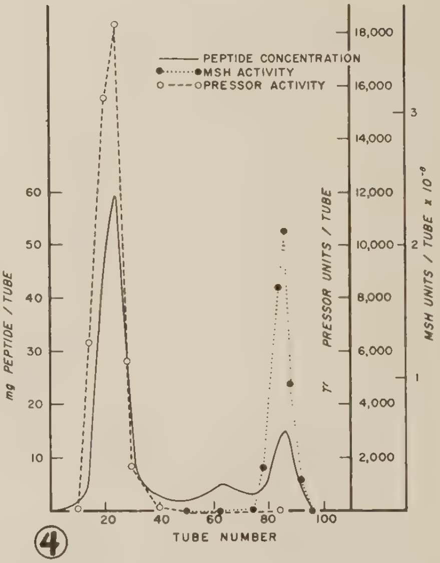

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FIG. 1. Separation of α- and β-MSH on CMC. 20 μl aliquots used for Folin-Lowry analysis.

FIG. 2. Chromatography of β-MSH on DEAE. 20 μl aliquots were analyzed with Folin-Lowry reagent.

FIG. 3. Separation of α-MSH and vasopressin on CMC. Folin-Lowry color obtained with 50 μl aliquots.

FIG. 4. CCD of lysine vasopressin and α-MSH.

For complete separation of pressor and MSH activities, countercurrent distribution was the method of choice. The basic peptide fraction from CMC was subjected to 100 transfers in all glass CCD apparatus, using the system of n-Butanol, 0.03 M p-toluene sulfonic acid, with 10^{-3} M EDTA. The material (1 g) was dissolved in 20 ml of lower phase and placed along with 20 ml upper phase in the first 2 cells of the CCD apparatus. Fig. 4 shows that there was a complete separation of lysine vasopressin and α-MSH, K values differing greatly in this system, 0.32 *vs.* 6. Tubes 15-30 were pooled for recovery of vasopressin and 76-98 for α-MSH. Material in upper phase was displaced into the lower phase by addition of 3-5 vol. of benzene and was deionized by passage through Amberlite CG-45 resin in acetate form. Yields were 500 mg of lysine vasopressin ($\geqq 300$ U/mg) and 200 mg of α-MSH (1.3-1.5 MSH U/mg, pressor activity 0.2 U/mg). Data from experiments with synthetic materials led us to believe that this minimal pressor activity may be inherent to the molecule of α-MSH.

α-MSH could be freed of small amount of fast moving contaminant by re-chromatography on CMC. Potency of final material was 1.5-2.0 x 10^7 U/mg. This α-MSH preparation was homogeneous on electrophoresis and chromatography in $BuOH$: $HOAc$:$H_2O = 4:1:5$, $R_f = 0.6$.

Tryptophan was detected spectrophotometrically and only the other 11 amino acids of α-MSH were found after acid hydrolysis. Overall recovery of α-MSH after precipitation with organic solvents(6), CMC chromatography and countercurrent distribution was 40% of total MSH activity in Pitressin Intermediate.

Summary. α- and β-MSH were isolated on preparative scale with high recovery of starting activity. The initial step in purification of both hormones is chromatography on carboxymethylcellulose. β-MSH is further purified (4-5 x 10^6 U/mg) by use of DEAE cellulose. α-MSH was not completely freed of vasopressin and other contaminants by zone electrophoresis on large vertical column. Countercurrent distribution of concentrate of vasopressin and α-MSH, yields α-MSH with a potency of 1.5 x 10^7 U/mg, free of vasopressin.

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Studies of Gastrointestinal Viral Flora of Cholera Patients.*† (25813)

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Pathogenesis of cholera is poorly understood. Presence of the organism in the environment or even in the individual person does not necessarily result in the clinical disease(1). In the disease state, cholera vibrio appears to exert a profound effect on the gastrointestinal tract without entering into the tissue deeply enough to be found in the blood stream(2). Many theories have been postulated to explain the pathogenesis of this disease. Among them has been presence of symbiotic or synergistic microorganisms including viruses. With the newly developed tissue culture technics for isolation of virus, and the discovery that large numbers of virus inhabit the gastrointestinal tract, a study was under-

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† Opinions and assertions contained herein are those of the authors and are not to be construed as official or as reflecting views of Navy Dept. or naval service at large.

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taken of viral flora of the gastrointestinal tract of cholera patients as revealed by culture in monkey kidney and HeLa cell tissue cultures. This study was carried out during cholera epidemic in Bangkok, Thailand in 1958 and 1959.

Materials and methods. Stool specimens were collected from patients with cholera, some of which were being treated by the NAMRU-2 team(3), and others from the general ward at Chulalongkorn Hospital, Bangkok in 1958. All patients had severe classical cholera with rice water stools and severe dehydration. In 1959 patients with cholera at Municipal Infectious Disease Hospital in Bangkok, were cultured. Many of these patients had only mild clinical symptoms, but cholera organisms were isolated from each(1). During study of epidemiology of the cholera epidemic in 1958, rectal swab specimens were collected from family members of cholera patients(4). None of these family members were ill, and cholera organisms were isolated from only 3 out of 243. Virus isolation attempts were made on 57 of these specimens collected from persons whose stool culture did not contain cholera organisms. All specimens to be cultured for virus were packed in dry ice and sent to NAMRU-2. Technics used for virus isolation and identification have been reported(5). Primary monkey kidney cultures were prepared with trypsin from Taiwan monkeys (*Macaca cyclopis*)(6). Eagle's medium(7) with 5% calf serum was used for both tissues. Cultures in HeLa cells were observed 40 days prior to being declared negative. Cultures of cholera patients' specimens in monkey kidney cells were observed 34 days prior to being declared negative. Specimens from well persons were observed in monkey kidney tissue culture only through one passage of variable length. Isolates were identified by neutralization tests with the following type specific antiserums; adenovirus 1-10, poliovirus 1-3, Coxsackie virus A9 and B1-5, ECHO virus 1-14.

Results. A total of 6 viruses was isolated from 44 patients with proven cholera. These included 3 adenoviruses and 3 enteroviruses. Serologic type identifications were made on 3 of these viruses. One was an adenovirus type 4, one Coxsackie type B3 and the other ECHO type 9. The one isolation in 1959 from among 17 mild cases was ECHO type 9 virus which came from an 18-month-old child. From among 57 well persons cultured, 12 viruses were isolated. Five were adenoviruses and 7 enteroviruses. One of the adenoviruses was type 6, and 6 of the enteroviruses were ECHO viruses, 2 type 4 and one each type 5, 6, 12 and 14. The remaining isolates produced cytopathogenesis typical of their group on appropriate cell type, but could not be neutralized by antiserums available in this laboratory. They are presumed to be adenoviruses other than types 1-10 and ECHO viruses other than types 1-14.

TABLE I. Isolation of Viruses from 1958 Cholera Patients and Non-Ill Family Contacts by Age Group.

		0-10 yr		11-50 yr	
		No.	%	No.	%
Cholera patients	Adenovirus	1/9	11	2/18	11
	Enterovirus	2/9	22	0/18	0
"Control" persons	Adenovirus	2/25	8	3/32	9
	Enterovirus	7/25	28	0/32	0

Nine cholera patients cultured in 1958 were children to age of 10, the rest were adults. Table I shows a comparison of isolation percentage of adenoviruses and enteroviruses from cholera patients and "control" persons by age group for the 1958 cases. From adults, only adenoviruses were isolated. From children, adenoviruses and enteroviruses were isolated from both groups. The cholera patient with Coxsackie B3 was 17 months old. The ECHO viruses were isolated from children under 5 years of age with the exception of 1 ECHO 4 from a 10-year-old.

Discussion. This study revealed no evidence of any association between viruses of the enterovirus or adenovirus groups with the clinical syndrome of cholera. There was a low percentage of isolation of viruses from cholera patients, and there was no tendency for any one type of virus to be isolated. Viruses were isolated as frequently from well persons as from cholera patients. Although family contacts without evidence of illness could not be considered a suitable control group, they at least give some idea of distribution of these types of viruses in the population at the same time, for comparison by age group with the cholera patients.

There was no evidence that disturbance of the gastrointestinal tract by cholera increased virulence of the adenoviruses and enteroviruses isolated. All cholera patients from whom virus was isolated recovered promptly after adequate fluid and electrolyte replacement therapy, and no other illnesses were diagnosed during the convalescent period.

Summary. Virus isolation studies on rectal swab specimens of cholera patients were carried out in tissue cultures during the Bangkok, Thailand cholera epidemic of 1958 and 1959. An adenovirus or enterovirus was isolated from 6 of 44 patients with proven cholera. It was concluded that viruses which can be isolated in monkey kidney and HeLa cell tissue cultures were not involved in the cholera syndrome.

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Response of Peritoneal Exudate Cells to *Brucella melitensis.* Influence of Nature of Inflammatory Irritant.* (25814)

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For many years there has been increasing interest in the use of mononuclear cells from rabbit and guinea pig peritoneal exudates as a tool for study of specific and non-specific resistance to bacterial and viral infections. One difficulty in comparing findings of different inevstigators rests principally with use of different animal species as sources of the monocytes and with inoculation of different inflammatory agents into the peritoneal cavity. This subject has recently been reviewed (1). To learn whether the nature of the irritant influences activity of cells which accumulate in response to intraperitoneal inoculation into the rabbit, a comparison has been made in which such irritants as light mineral oil, sodium caseinate, dextran, glycogen and killed Brucella cells were used. Responses of monocytes from normal and Brucella-immune rabbits to parasitization by *Brucella melitensis* were studied by methods described(1).

Methods and materials. Irritants. "Klearol" (obtained from L. H. Butcher Co., San Francisco). Casein (supplied as sodium caseinate by Mann Assayed Biochemicals, approximately 90% protein). A 2.5% solution of the protein in saline was employed. Glycogen (Nutritional Biochemicals). Dextran (Cutter Labs) in the form of 6% solution for intravenous injection. *Br. melitensis,* scraped off Albimi agar slants after 3 days incubation at 37°C washed twice and resuspended in saline. Monocytes from adult rabbits, both normal and immunized, were injected with one of the inflammatory agents. For example, either sterile Klearol, 1.2% (W/V) solution of sodium caseinate, 6% Dextran (W/V), 0.1 mg % (W/V) glycogen or a formalin-killed, saline suspension (10^9/ml) of a virulent strain

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TABLE I. Responses of Peritoneal Exudate Cells to *Brucella melitensis.*

		Cell counts				% degeneration		
Stimulant	Type of cell	0	24	48	96	24	48	96
Oil	Normal, non-parasitized	195	190	194	267	3	0	0
	" parasitized	118	84	68	61	29	42	48
	Immune, non-parasitized	238	237	237	236	.5	0	.5
	" parasitized	432	430	432	431	.5	0	.3
Caseinate	Normal, non-parasitized	263	262	258	264	.4	2	0
	" parasitized	392	392	389	487	0	.8	0
	Immune, non-parasitized	350	349	353	310	.3	0	0
	" parasitized	82	100	100	100	0	0	0
Glycogen	Normal, non-parasitized	576	576	571	450	0	1	22
	" parasitized	373	370	371	300	1	0	20
	Immune, non-parasitized	155	160	168	123	0	0	27
	" parasitized	166	111	102	55	34	39	67
Dextran	Normal, non-parasitized	1062	1057	1040	1296	.5	2	0
	" parasitized	732	732	715	803	0	3	0
	Immune, non-parasitized							
	" parasitized							
Brucella melitensis	Normal, non-parasitized	442	438	444	438	1	0	1
	" parasitized	253	153	150	90	39	40	64
	Immune, non-parasitized	1349	1341	1330	1380	.6	1	0
	" parasitized	1320	1310	1325	1389	.8	0	0

of *B. melitensis* was injected intraperitoneally in 50 ml. Six days later monocytes were collected by washing peritoneal cavity with 200 ml Tyrode solution. The cell suspension was filtered through gauze and sedimented. Cells were redispersed in Tyrode solution containing 0.25 g % trypsin (Difco, 1:250). After 25 minutes at 25°C the cells were washed 3 times in chilled Tyrode solution. After a final centrifugation the packed cells were suspended in fresh homologous serum. Cells were parasitized by mixing a sufficiently concentrated saline suspension of a strain of *B. melitensis* with monocytes in paraffin-lined bottles. The mixtures were centrifuged, then held 1 hour at 4°C to allow 25 to 35% of monocytes to become infected. Supernatant fluid was then removed as completely as possible, sediment resuspended in small volume of medium consisting of 40% homologous rabbit serum and 60% Tyrode solution to contain approximately 15 monocytes/mm^3. Assembly and preparation of Mackaness cell culture chambers has been described(1). To obtain monocytes and sera from immune rabbits, the animals were inoculated subcutaneously 3 months earlier with 10^9 live cells of *B. melitensis* strain, Rev. I(2).

Results. Monocytes from normal as well as from Brucella-immune rabbits were parasitized with living, attenuated, vaccine strain of *B. melitensis,* Rev. I. Survival and maintenance of parasitized cells were recorded daily as described earlier(1). The results from 3 Mackaness chambers were gathered to measure response of each type of cell (Table I). Data are taken from representative experiments, of which 9 replicates were carried out in detail. Data from all 9 experiments were in excellent agreement, showing less than 0.5% variation. Table I shows markedly different responses of monocytes induced either by oil or *B. melitensis* suspension on the one hand, and casein, dextran and glycogen on the other. Responses of monocytes induced by oil or by killed *B. melitensis* were hardly distinguishable. Furthermore, monocytes of immune and normal animals responded to parasitization by living *B. melitensis* in a manner dependent upon immune or susceptible state of animal rather than upon the nature of inflammatory agent (oil or bacterial suspension).

In contrast, monocytes of normal rabbits appearing in response to casein and dextran behaved towards the parasitizing organism as if they came from immune rabbits, *i.e.,* monocytes induced by casein and dextran were not destroyed by a strain of *B. melitensis* cytolytic for those monocytes induced by oil or bacterial suspension.

Glycogen on the other hand stimulated a paradoxical reaction in monocytes, in which the "immune" monocyte (in presence of homologous serum) was more susceptible to cytolytic action of the parasitizing bacillus than was the monocyte from non-immune animals. Table I also shows that glycogen-stimulated, parasitized monocytes from immune animals were destroyed more rapidly than parasitized monocytes from nonsensitized animals. Use of glycogen to induce peritoneal inflammation caused a strong tendency to peritoneal bleeding and monocytes taken from such animals were very often characterized by extreme fragility. When sufficient numbers of animals were used, it was possible to obtain a few in which such bleeding into the peritoneum did not occur; these animals yielded monocytes which appeared to be hypersensitive to cytolytic action of Brucella.

It was of interest to study survival values of mononuclear cells which appeared in response to various irritants. This quality is of particular importance in experiments on growth and multiplication of intracellular parasites and the variables affecting monocytic destruction of parasites(1).

To overcome the difficulty of removing monocytes from glass surfaces to which they had adhered, plasma clot was employed as the cultivating surface. For this purpose, blood was withdrawn from a rabbit and placed in a tube containing 0.1 mg of heparin/20 ml blood. Blood cells were immediately spun down and 0.5 ml of plasma was dispensed into several Carrel flasks. Simplastin (0.1 ml) was prepared by resuspending contents of 1 vial of commercial preparation (General Diagnostics Div., Warner-Chilcott Co.) in 8 ml distilled water. The clot formed very quickly after addition of Simplastin.

To each Carrel flask containing the plasma clot were added 1 x 10^4 mononuclear cells prepared as described above. Varying periods of time thereafter, duplicate flasks were treated with 0.25% trypsin for 30-60 minutes to remove the clot. A viable count of mononuclear cells was performed in a haemocytometer after cell suspension had been exposed to 0.001% (W/V) aqueous eosin.

Increases in cell population occurred for 6-9 days in the exudate induced by Klearol and glycogen: cells stimulated by dextran deteriorated under identical conditions within 72 hours.

Monocytes induced by oil were maintained under conditions of the Carrel flask plasma clot method for 4 months. This was done by treating the clot with trypsin, washing freed monocytes 3 times in Tyrode solution and replanting the cells onto the plasma clot at 10 day intervals.

Conclusion. Studies on mononuclear cell responses to *B. melitensis* require that attention be paid especially to the nature of inflammatory agent itself when interpreting behavior of the mononuclear cell. The inflammatory substance may so alter mononuclear response as to reverse cytolytic susceptibility of monocytes from normal and immunized animals.

Summary. A comparison has been made of the effect of several irritants on response of mononuclear cells from rabbit's inflamed peritoneum. Animals injected intraperitoneally with mineral oil or a suspension of killed *B. melitensis* stimulated accumulation of peritoneal monocytes whose behavior was identical towards living *B. melitensis, i.e.*, brucellae were cytolytic for normal rabbit monocytes but not for immune rabbit monocytes. Dextran and casein stimulated exudation of relatively insusceptible monocytes. Glycogen induced exudation of a relatively fragile monocyte, the susceptibility of which to Brucella parasitization was increased by the *immune state* of host animal.

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Serological Relationship of Trachoma, Psittacosis and Lymphogranuloma Venereum Viruses.* (25815)

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The agents of psittacosis-lymphogranuloma venereum and trachoma-inclusion conjunctivitis have been classified together in the family *Chlamydiaceae* on morphological grounds, but placed in separate genera because of the assumption that their cultural characteristics differed(1). Rake *et al.*(2), employing crude lymphogranuloma venereum antigen, reported presence of complement fixing antibodies to the group antigen of psittacosis-lymphogranuloma venereum (P-LV) in a small number of sera from trachoma patients. Kornblueth and co-workers(3) tested sera from 104 patients suffering from various stages of trachoma with similar antigen and found that 24% gave a positive serological reaction. Other workers employing trachoma antigens prepared from epithelial scrapings of patients with active infection reported complement fixing antibodies in sera of patients with trachoma, lymphogranuloma venereum and ornithosis(4). Recent isolation of trachoma virus in yolk sac of embryonated eggs(5-10) provided the opportunity for more definitive studies of the relationship of trachoma to the P-LV group. Most trachoma virus strains isolated in different parts of the world contained the P-LV group antigen(6,7,9,10). Use of trachoma virus prepared as group antigen for diagnosis of trachoma proved disappointing, and we recently reported preliminary results with specific "purified" elementary body (PEB) trachoma virus antigen, which measures complement fixing antibodies in 33 to 62% of persons on Taiwan with clinical diagnosis of trachoma(11). The work reported here makes use of this specific purified antigen along with group antigens to study the relationship of trachoma virus to psittacosis and lymphogranuloma venereum viruses in the complement fixation test.

Materials and methods. Trachoma virus, strain TW-29(11), in 16th egg yolk sac passage was used. Psittacosis virus, strain 6BC, has been maintained in the laboratory of one of the authors since 1947(12). Lymphogranuloma venereum (LGV) virus, strain JH, was obtained from American Type Culture Collection by Dr. F. B. Gordon who made 2 passages. Psittacosis and LGV viruses were brought to NAMRU-2 as frozen 20% yolk sac suspensions. *Antisera.* Human sera were used throughout, and were stored in frozen state. Trachoma sera with relatively high CF titer when tested with PEB antigen were chosen for study. Trachoma sera from Egypt were obtained in cooperation with Dr. Abdel-Fattah M. Mohamed at Memorial Inst. for Ophthalmic Research, Giza, Cairo. The 2 LGV sera pairs were obtained from Virus Dept., Walter Reed Army Inst. of Research. These sera were lyophilized for transport to NAMRU-2. Two fresh sera specimens were obtained on Taiwan from active cases of LGV. Psittacosis antisera from 3 cases diagnosed during 1959 were obtained from Dr. Helen L. Casey, Communicable Disease Center, U. S. PHS. A fourth pair of psittacosis sera was available from a person who contacted laboratory infection with the 6BC strain in 1947. *Preparation of antigens.* Six- to 8-day-old chick embryos were inoculated into the yolk sac with predetermined virus inoculum to produce a maximum yield of virus. Yolk sacs from all embryos surviving 3 days or longer were harvested just before or after death, smeared, cultured and stored at −65°C. Only sterile membranes, heavily infected with virus by microscopic examination using Macchia-

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vello's stain, were used for antigen preparation. PEB antigens were prepared according to technic developed in this laboratory(11). Twenty % buffered saline suspensions of yolk sac tissue adjusted to pH 8.0 with 1% sodium carbonate were incubated with 0.1 ml of 0.1% trypsin/ml of suspension at 37°C for 3 hours. Trypsin was added and pH adjusted at end of first and second hours of digestion. Only the pH was adjusted at end of third hour of incubation. Then following 2 cycles of low speed centrifugation to remove floating fat and large particles, the virus was sedimented 3 times at 10,000 rpm for 30 minutes in No. 40 head of Spinco model L centrifuge. The resuspended elementary bodies were further purified by precipitation of foreign material with polymyxin B, 100 γ/ml, and elution of any accompanying virus from the precipitate. Formaldehyde USP was added to 0.02% concentration. PEB antigens were adjusted in volume to 40% original yolk sac suspension. These preparations had C. F. antigen titers of 1:12 to 1:24 in presence of 2 units of homologous antisera. Boiled phenolized antigens were prepared after method of Nigg, Hilleman and Bowser(13). Phenol to 0.5% concentration was added to 10% suspensions of infected yolk sacs, and incubated at 37°C for 4 weeks, then placed in boiling water for 20 minutes. After centrifugation at 1500 rpm for 10 minutes the straw colored supernatant was removed and used as group antigen. The complement fixation test used in the current work was the modified Kolmer technic employing optimal proportions method of Friedewald, as described by Rosenbaum and Woolridge(14). In all tests 2 units of antigen and 2 full units of complement were mixed with serial dilutions of serum and incubated overnight at 5° C. The following day after the tubes were warmed at 37°C for 10 minutes, 2 units of hemolysin and 2% sheep red blood cells were added. The materials were again incubated at 37°C for 30 minutes after which the tests were read. Total volume of each tube was 1.5 ml. The highest serum dilution in which 3+ fixation occurred (75%) was taken as endpoint and reported as reciprocal of original serum dilution.

Results. The Table records results of complement fixation tests. All convalescent sera from psittacosis reacted with each of the 3 group antigens and with PEB preparations of both psittacosis and LGV. Somewhat higher antibody titers were found with PEB antigens and trachoma virus group antigen than with psittacosis and LGV group antigens, but the differences in titer were small. LGV sera reacted more irregularly but followed in general the same pattern of reaction as psittacosis sera. No psittacosis or LGV sera fixed complement with trachoma PEB antigen except the Mitchell serum at 1:8 dilution. This serum had been stored for 9 years and contained a slight precipitate. On the other hand, many sera from trachoma patients reacted only with PEB trachoma virus antigen. None of these serums reacted with PEB antigens of psittacosis and LGV. The group antigens reacted only with trachoma sera having highest antibody titer and then in low titer. Trachoma and psittacosis virus group antigens reacted with trachoma sera more frequently than LGV antigen. Serum from Taiwan patient #7182 reacted with all antigens. This patient came to the clinic to be treated for lymphogranuloma venereum and had not only a large bubo but also clinical trachoma in active stage.

Discussion. Although many studies on antigens of the P-LV group have been undertaken to obtain a serologic procedure for identifying individual members, no simple test has been developed(15). In the present work, infections with trachoma virus could be clearly differentiated serologically from those caused by psittacosis and LGV viruses by use of PEB preparations as antigen in the complement fixation test. However, with similar purified antigens of psittacosis and LGV viruses no differentiation was possible between sera from patients with these 2 diseases. Therefore, the method of purifying elementary bodies does not provide a new tool to separate viruses of the P-LV group but shows that trachoma virus contains a specific antigen different from other human pathogens of the group.

Summary. Sera from patients with trachoma, psittacosis and lymphogranuloma ven-

TABLE I. Complement Fixation Antibody Titers of Serums from Patients with Psittacosis, Lymphogranuloma Venereum and Trachoma with Purified Elementary Body (PEB) and Boiled Phenolized Group Antigens.

		Complement fixation antibody titer					
		PEB antigen			Group antigen		
Disease	Serum	Trach	Psitt	LGV	Trach	Psitt.	LGV
Psittacosis	59-0661 Spec. 1	0	0	0	0	0	0
	Idem 2	0	64	32	32	16	16
	" 3	0	32	32	32	16	16
	59-0855 Spec. 1	0	32	32	32	8	16
	Idem 2	0	128	64	32	16	16
	" 3	0	128	128	32	16	16
	59-1124 Spec. 1	0	32	16	16	8	8
	Idem 2	0	128	64	32	32	16
	E. J. April 1947	0	0	0	0	0	0
	Sept. "	0	64	64	64	32	16
LGV	Mitchell 12- 8-50	0	0	0	0	0	0
	4-11-51	8	128	128	64	16	32
	McC. 6-18-53	0	0	0	0	0	0
	7-15-53	0	64	64	8	0	0
	Taiwan pt. #6183	0	64	128	32	128	128
Trachoma	Taiwan pt. # 363	16	0	0	0	0	0
	Idem #1081	16	0	0	0	0	0
	" #1167	64	0	0	16	8	8
	" #1079	64	0	0	16	8	0
	Egypt pt. #14	32	0	0	8	0	0
	Idem #17	32	0	0	16	16	0
	" #11	64	0	0	16	16	0
	" #15	16	0	0	0	0	0
LGV and trachoma	Taiwan pt. #7182	64	128	256	64	64	64

Results are expressed as reciprocals of original serum dilution. 0 = <8.

ereum were tested in the complement fixation test with both "purified" elementary body antigens and boiled phenolized group antigens made with each of the 3 viruses. Group antigen prepared with trachoma virus reacted similarly to group antigens made with psittacosis and lymphogranuloma venereum viruses. The trachoma elementary body antigen reacted only with sera from trachoma patients. Psittacosis and lymphogranuloma venereum elementary body antigens failed to react with trachoma serum but did react about equally with sera from both psittacosis and lymphogranuloma venereum patients. It is concluded that trachoma virus contains the group antigen of the P-LV group and in addition a specific antigen which permits its serologic differentiation from the agents of psittacosis and lymphogranuloma venereum.

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Infection of Hamsters with Para Influenza 3 Virus. (25816)

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A property shared by many myxoviruses is their capacity for multiplication in lungs of certain rodents(1,2). Many of the genetic factors and quantitative aspects of experimentally induced infections with influenza viruses have been described(3). With extensive use of tissue culture for virus isolation, a group of myxoviruses with common properties has recently been recognized(4,5). These viruses were designated as para influenza viruses 1, 2, and 3 to indicate their distinctness from influenza viruses. Because of their importance in the etiology of childhood respiratory illness, and of their potential importance in adult respiratory disease, experimental animal hosts were sought in which *in vivo* virus-host relationships could be studied(6). The present study is concerned with occurrence and quantitative aspects of pulmonary infection in hamsters with the type 3 para influenza virus.

Methods and materials. Virus. The C 243 strain of para influenza 3 virus, isolated in 1957 from a child with pneumonia(5), was passaged 7 times in rhesus monkey kidney cultures and twice in Salk monkey heart cultures. A stock pool of virus, prepared by combining equal volumes of infected monkey heart culture fluid and skimmed milk, was stored in glass sealed ampules in a CO_2 chest. *Inoculation of hamsters.* One-month-old, recently weaned, golden hamsters of both sexes, weighing 60 to 70 g were used. One-tenth ml of varying dilutions of tissue culture fluid or lung suspension was inoculated intranasally under ether anaesthesia. Inoculated animals were housed in groups of 2 or 3 in shoebox-sized containers in semi dark room. Control animals were inoculated with sterile normal lung suspension and housed similarly. At various intervals following inoculation, hamsters were lightly anaesthetized with ether and exsanguinated. Lungs were removed aseptically, washed twice in Hanks' balanced salt solution, and a 10% suspension prepared in 50% skimmed milk in Hanks' solution by grinding in mortar with alundum. Lung suspensions were stored at –60°C until titered. *Titration.* Decimal dilutions were prepared in Hanks' solution and 0.2 ml was inoculated into each of 2 rhesus monkey kidney cultures. These cultures were maintained in Eagle's basal medium without serum. Seventy-two hours following inoculation, cultures were examined for evidence of infection by the hemadsorption technic. Cultures were washed twice with Hanks' solution and 1 ml of 0.1% suspension of guinea pig red cells was added. Following 20 minutes incubation at 4°C they were examined for presence of erythrocytes adsorbed to the tissue culture sheet. TCD_{50} (50% tissue culture dose) was calculated by the method of Reed and Muench(7). *Serology.* Complement fixation tests were performed by modification of the method of Bengston(5). Complement fixing antigens for various myxoviruses were prepared as described previously (8).

Results. Para influenza 3 virus was carried through 15 consecutive intranasal passages in hamsters. The inoculum of tissue culture virus which initiated the passage series con-

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TABLE I. Multiplication of Para Influenza 3 Virus in Hamster Lung.

	Quantity of virus recovered from lungs at indicated time after inoculation—$\log_{10}$			
	Exp. 1		Exp. 2	
Hr after intranasal inoculation	Tissue culture virus* Inoculum = 4.7	Hamster lung passage 6 virus† Inoculum = 4.2	Tissue culture virus Inoculum = 4.7	Hamster lung passage 8 virus‡ Inoculum = 4.2
1	<2.4	<2.4	<2.4	<2.4
6	<2.4	<2.4	<2.4	<2.4
12	4.1§	2.8	2.4	3.1
24	5.4	4.8	4.9	4.9
36	5.9	5.2	6.1	6.1
48	5.8	5.9	6.4	6.1
72	6.2	5.8	———Not done———	

* 7 passages in tissue cultures derived from monkey kidney followed by 2 passages in monkey heart culture.

† 6 *in vivo* hamster passages initiated with tissue culture virus.

‡ 8 *Idem*

§ Mean of values obtained from 2 hamsters.

tained $10^{5.4}$ TCD_{50}. Hamsters were sacrificed after 48 hours and 10% suspension of lung tissue served as inoculum for the next passage. A 48-hour interval was selected for the passage series since growth curve study indicated that near maximal levels of virus were present at that time (Table I). Virus content of lungs ranged between $10^{5.9}$ and $10^{6.9}$ TCD_{50} and the quantity present during early passages did not differ from that during later passages. No respiratory symptoms were noted, no deaths occurred, and no gross lung lesions were observed.

Para influenza 3 virus was not detected in lungs 1 or 6 hours following inoculation of $10^{4.7}$ TCD_{50} of tissue culture grown virus. At 12 hours virus was present at a low level and increased in titer over the next 24 hours to reach a high level at 36 hours (Table I). These findings, together with results of 15 serial hamster passages, indicate that para influenza 3 virus possesses capacity for growth in the hamster lung.

Not shown in Table I is the finding that levels of virus comparable to those recovered at 48 and 72 hours were present 96 hours after intranasal instillation. At 120 hours, small quantities of virus averaging 10^{3} TCD_{50} were present in the lung, while at 144 hours virus could not be detected. Lung lesions were not observed 6 days following virus inoculation.

The pattern of growth of 6th and 8th hamster passage virus in hamster lung was compared with that of tissue culture virus from which it was derived. This comparison (Table I), was performed to determine if variants with a higher level of growth or a more rapid pattern of replication were selected during animal passage. No significant difference was evident in the growth curves, suggesting that tissue culture virus population contained virus particles possessing the properties for optimum replication in the hamster lung.

Quantity of virus required to initiate infection in the hamster lung was determined (Table II) for virus grown only in tissue culture and for virus which had undergone 5 subsequent passages in hamster lung. Approximately 1 tissue culture infectious dose of tissue culture virus was sufficient to infect hamsters by intranasal route. After 5 hamster lung passages, the quantity of virus required to initiate infection of the hamster did not decrease. Tissue culture virus was thus as capable of infecting hamsters as virus which had been passaged in this animal. These findings suggest that selection of variants with increased infectivity for hamsters did not occur during hamster passage. It appears that virus from human source which had been grown only in tissue culture possessed both optimum growth characteristics and infectivity for the hamster lung.

Six hamsters were inoculated intranasally

TABLE II. Quantity of Para Influenza 3 Virus Required to Infect Hamsters by Intranasal Route.

Virus propagated in					
Tissue culture*			Hamster lung†		
Inoculum TCD_{50}	No. hamsters infected/No. inoculated	TCD_{50} recovered from lungs,‡ $\log_{10}$	Inoculum TCD_{50}	No. hamsters infected/No. inoculated	TCD_{50} recovered from lungs,‡ $\log_{10}$
320	3/3	5.4, 5.9, 5.9	500	3/3	4.9, 5.4, 5.9
32	3/3	3.9, 4.9, 5.9	50	2/3	2.4, 2.4
3.2	2/3	2.4, 3.4	5	0/3	
.3	1/3	3.4	.5	0/3	
.03	0/3				

* Monkey kidney culture passage 7, monkey heart culture passage 2.

† 5 *in vivo* hamster lung passages initiated with above described tissue culture virus.

‡ Lungs tested 48 hr after intranasal inoculation of virus.

with $10^{4.7}$ TCD_{50} of virus and bled 3 weeks later to study their serologic response to para influenza 3 infection. When tested by complement fixation, pooled serum from these animals had a titer of 1:1280 with para influenza 3 tissue culture antigen and 1:20 with para influenza 3 soluble antigen. This serum failed to react at a 1:10 dilution with the tissue culture or soluble antigens of para influenza 1 (hemadsorption type 2 and Sendai), para influenza 2, or mumps.

Discussion. Previous studies on replication of myxoviruses in the rodent lung have dealt mostly with influenza in the mouse. Egg propagated and mouse adapted influenza viruses differed in their pattern of growth in the mouse lung. Strains of influenza A or B not adapted to the mouse were characterized by a lag phase during which multiplication did not occur and by production of a smaller quantity of virus in the lung compared to the adapted strains which did not exhibit a lag phase(3). Adaptation following mouse passage has been interpreted as selection and overgrowth of virus mutants which replicate to a high titer without a preliminary lag phase.

The present findings which describe properties of tissue culture propagated para influenza 3 virus in hamster lung, differ considerably from those just summarized for influenza virus in mouse lung. Without prior multiplication in hamster lung, para influenza 3 virus appears to possess the genetic properties for hamster infectivity and replication which characterize an "adapted" strain. Thus, tissue culture para influenza 3 virus resembled virus which had been passaged in hamster lung as regards rate of multiplication in lung, quantity of virus produced in lung, and quantity of virus required to initiate infection. In fact, one infectious dose of tissue culture virus as measured in that system was sufficient to infect hamsters by the intranasal route.

The finding that para influenza 3 virus multiplies to high titer in hamster lung may have practical implications in standardization and evaluation of vaccines. In addition to the usual antibody production test, vaccines could be assayed for potency by their effect upon virus multiplication in the lung when hamsters are challenged subsequent to vaccination.

Previous studies have established that para influenza viruses share common antigens(5,8). Of some importance is the significance of heterotypic antibody in resistance to, or modification of infection. A study of hamsters infected with one para influenza virus and challenged with a heterotypic virus may yield information pertinent to this problem. An investigation of this nature is currently in progress.

Summary. Hamsters were infected by the intranasal route with para influenza 3 virus. One tissue culture infectious dose was sufficient to infect hamsters. Quantity of virus produced in the lung was the same, $10^{5.9}$ to $10^{6.9}$, during infection with virus grown only in tissue culture or virus passaged in hamsters. Infected hamsters developed high levels of CF antibody specific for para influenza 3 virus.

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Method for Detecting Antiviral Agents on Paper Chromatograms. (25817)

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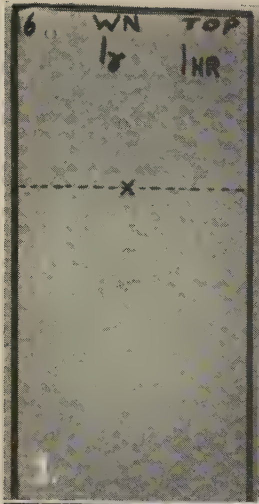
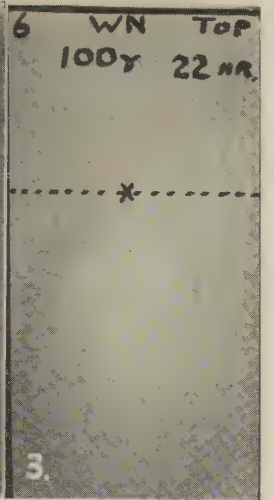
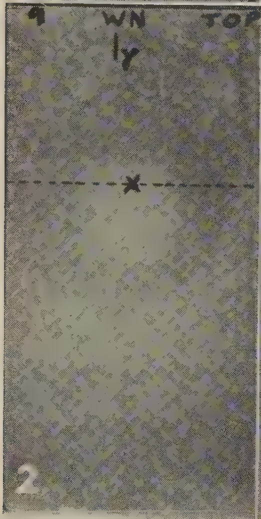
The procedure of placing paper chromatograms on the surface of agar seeded with bacteria to locate zones of antibiotic activity was first used to distinguish various components of a penicillin mixture(1,2). This method, now universally used in identification and purification of naturally occurring antibacterial agents, can also be used in a similar manner for characterization of antiviral agents. The following discussion describes an adaptation of the Dulbecco virus-plaque technic(3) to this purpose.

Materials and methods. Preparation of agar overlay, chick embryo cell cultures in Pyrex baking dishes, infection of these cultures with West Nile and vaccinia viruses, subsequent staining of cells to observe plaques, and detection of zones of virus inhibition have been described(4). *Antiviral test materials* used in our experiments include antibiotic W-112, extracted with ether from an unclassified streptomyces species culture and isatin-monothiosemicarbazone synthesized in our laboratories by Dr. Frank Villani. Antibiotic W-112 at concentrations as low as 0.1 μg produced significant inhibition of plaques formed by W. Nile, vaccinia, herpes simplex and Newcastle disease viruses. Isatin-monothiosemicarbazone inhibited only vaccinia virus plaque formation. Since this compound is also active in preventing death of mice infected with many lethal doses of vaccinia virus(5), it represents a model of an antiviral agent active both *in vitro* and *in vivo*. *Paper chromatographic* migration of antibiotic W-112 was obtained with partition systems using aqueous methanol as a stationary phase(6) on No. 1 Whatman paper at 20°C. Development time ranged from 1.1 to 3.2 hours. Solvent systems included system 6, a mixture of 300 ml toluene, 100 ml light petroleum, 300 ml methanol and 700 ml of water. Solvent system 9 was a mixture of 200 ml toluene, 800 ml light petroleum, 200 ml methanol and 800 ml of water. Analysis of isatin-monothiosemicarbazone was carried out on No. 1 Whatman paper at 20°C, using solvent system 16, a mixture of 120 ml n-butanol, 30 ml acetic acid and 50 ml of water, and solvent system 17 made up of 100 ml n-butanol, 100 ml pyridine and 100 ml water. After drying, the ethylene oxide sterilized paper chromatograms (10 x 34 cm) were cut in half and top and bottom portions placed for 5 min on separate agar overlays of virus infected cultures. This time period was adequate for diffusion of the antiviral agent into the agar. Longer time periods, however, frequently resulted in the cell sheet being destroyed, apparently by toxic residues left by chromatographic solvent systems. Position and orientation of the paper were indicated on bottom of baking dish. After removal of paper, baking dishes were sealed with Saran Wrap (Dow Chemical Co.), then incubated for 4 days at 36°C. The cell sheet was then







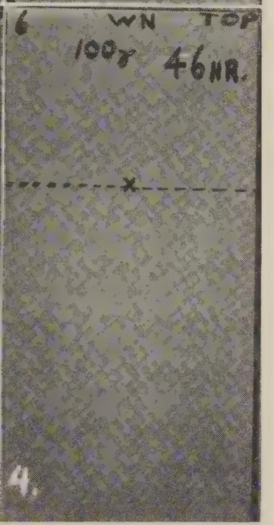


FIG. 1. A zone free of W. Nile virus plaques resulting from application of paper chromatogram to agar overlay 1 hr after virus infection. One μg of antibiotic W-112 was applied at ×. Migration of active component occurred after treatment with solvent system 6.

FIG. 2. Same procedure as for Fig. 1 except this paper chromatogram was treated with solvent system 9.

FIG. 3. Same procedure as for Fig. 1 except 100 μg of W-112 was used and paper applied to agar overlay culture 22 hr after virus infection.

FIG. 4. Same procedure as for Fig. 3 except paper applied 46 hr after virus infection.

stained with a second agar overlay containing indonitrotetrazolium chloride(4) and within a few hours plaques were readily observed.

Results. One μg of antibiotic W-112 was applied to chromatography paper and developed with solvent system 6 in a descending fashion for 1.6 hrs. Location of this antiviral agent could be observed by the resulting large, plaque-free zone in a baking dish culture infected with W. Nile virus (Fig. 1). When solvent system 9 was used, however, as a developing agent, the antibiotic migrated only very slightly (Fig. 2). In both cases the 4-day incubation period permitted the antibiotic to diffuse widely and, therefore, resulted in a plaque-free zone probably much larger than the original zone on the paper. Since W. Nile virus multiplies slowly, this no doubt also permits the antibiotic to diffuse for a considerable time prior to plaque formation. When 100 μg of W-112 was present in a paper chromatogram and this chromatogram was placed in contact with an agar overlay only 1 hr after virus infection, the resulting plaque-free zone covered the entire baking dish. It was found, however, that somewhat smaller zones were produced if the paper was applied to agar overlay culture 22 hours after virus infection (Fig. 3) and still smaller zones resulted if the paper was applied 46 hr after virus infection (Fig. 4). This suggests that size of zones of plaque inhibition can be controlled by proper choice of application time of paper chromatograms. When a very sensitive test is needed, the paper can be applied very soon after virus infection of overlay cultures. When sensitivity is not important but a more accurate determination of the area of antiviral activity on paper is needed, then application of the paper can be delayed. Each system would, no doubt, have to be worked out separately, depending on virus used and diffusion rate of antiviral agent.

Isatin-monothiosemicarbazone was chromatographed using previously described solvent systems. Migration of the active component was followed by using tissue cultures infected with vaccinia virus. With either solvent system 16 or 17 the active moiety migrated very rapidly and could be readily located on paper because of its bright yellow color. Subsequent development in agar overlay cultures showed that zones of plaque inhibition coincided with areas of yellow color on the paper. The plaque-free zones were, however, considerably larger than the color zones. It required

50 μg of isatin-monothiosemicarbazone to produce significant plaque-free areas. Smaller zones were observed with as little as 20 μg but none were seen when 10 μg was used. In no case were these zones equal in size to those produced by just 1 μg of antibiotic W-112; even though paper chromatograms of the synthetic compound were applied to the overlay cultures as soon after virus infection as possible.

Discussion. By adaptation of the Dulbecco plaque technic it is now possible to detect, assay and chromatograph antiviral antibiotics in a manner similar to antibacterial antibiotics. The same method is equally useful in chromatography of synthetic compounds such as isatin-monothiosemicarbazone. This synthetic antiviral agent was chosen for study because it is an agent unquestionably active both in virus infected animals and tissue cultures. Since this compound only inhibits plaque formation of vaccinia virus and not of W. Nile, herpes simplex and Newcastle viruses, this indicates that plaque-free zones are in this case a result of specific influences on the course of virus infection and not due to a subtle, general toxic effect on chick embryo cells.

Summary. A procedure has been developed whereby paper chromatograms of antiviral agents can be developed by applying them to virus infected, agar overlay, chick embryo cell tissue cultures. Plaque-free zones are produced, thus indicating the area on paper chromatogram where the active material can be located. Using antiviral substances antibiotic W-112 and isatin-monothiosemicarbazone, W. Nile and vaccinia viruses, and 4 chromatographic solvent systems, it was possible to localize the biological activity of these antiviral agents. As little as 1 μg of antibiotic W-112 produced large plaque-free zones when paper chromatogram was applied to the overlay culture 1 hr after virus infection. When 100 μg of same material was used, large plaque-free areas resulted even when the paper was applied 46 hr after virus infection. Isatin-monothiosemicarbazone at 50 μg level not only produced a plaque-free zone but also produced a yellow spot on paper chromatogram that coincided exactly with the zone of antiviral activity.

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Reserpine and Thyroid Activity in Fowls.* (25818)

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Tranquilizers have been suggested for use with poultry because of their stress ameliorating properties with possible beneficial effect on egg production, growth and feed efficiency(1, 2). Data are available, however, indicating that reserpine has an anti-thyroid effect(3,4). If this were true of poultry, administration of reserpine would then be contraindicated for purposes of sustained tranquilization where normal thyroid function is desired. Since data are extremely limited, it was of interest to study effects of reserpine in fowls using direct measurements of thyroid function *viz.,* thyroidal-I^{131} release rate and thyroxine secretion rate.

Materials and Methods. Adult New Hamp-

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TABLE I. Effect of Varying Levels of Reserpine on Net Release of I^{131} (k′ ′4) from Thyroid Gland and Body Weight in Adult New Hampshire Chickens. 10 chickens/series.

Reserpine dose, μg/100 g/day	I^{131} release rate with Tapazole (k′ ′4)	I^{131} release rate with Tapazole and reserpine	% change in body wt during reserpine administration
10	.0079 ± .0001	.0081 ± .0002*	+ 1.1 ± .423
20	.0092 ± .0009	.0090 ± .0004	− 1.0 ± .321
30	.0101 ± .0018	.0072 ± .0009†	− 8.2 ± .689†
50	.0089 ± .0004	.0044 ± .0008‡	−15.4 ±1.826‡
100	.0099 ± .0010	.0029 ± .0001‡	−18.0 ±2.101‡

* Stand. error. † Significant at $p < .05$. ‡ Significant at $p < .01$.

shire chickens were used, subjected to diurnal temperature variations. Pigeons were housed in chamber at 78 ± 1°F. 50-75 μc I^{131} were injected subcutaneously into chickens and 24 hours thereafter external thyroid counts were made by placing birds in weighing funnel(5). In pigeons, 20 μc I^{131} were injected subcutaneously and thyroidal-I^{131} measurements were made at 24-hour intervals by placing pigeon in aluminum funnel with thyroid region approximately 15 cm over scintillation probe (6). *Determination of thyroidal-I^{131} release rate.* Thyroidal-I^{131} release rate was determined in 5 groups of 10 adult New Hampshire chickens. Daily thyroid counts were made for 11 days commencing 24 hours after I^{131} injection. Tapazole was administered as 0.1% of ration to prevent recycling of I^{131} from metabolized hormone-I^{131} which would otherwise mask true release of thyroidal-I^{131}. Beginning 6 days after I^{131} injection, birds in each group received 5 daily subcutaneous injections of varying amounts of reserpine, 10, 20, 30, 50, or 100 μg/100 g body weight/day. Thyroidal-I^{131} release curves were constructed by plotting per cent dose against time in days. Hourly rate of release of thyroidal-I^{131} (k′ ′4) was calculated from slope of I^{131} release curve (7). *Determination of feed intake and body weight in pigeons.* Pigeons were housed in individual cages wrapped with paper, except at top, to prevent wastage of feed. Feces was separated from feed every day before computing amount of feed consumed by individual pigeon. Pigeons were weighed daily and at least 12 days were allowed to elapse before being included in investigation. *Determination of thyroxine secretion rate (TSR).* 15-18 adult common pigeons comprised a group and were injected with citric acid (1 μg/day) as a placebo, or reserpine in varying amounts (5, 10, 15, or 30 μg/100 g body weight/day) 48 hours after I^{131} injection until TSR was estimated. Seven days after I^{131} injection, pigeons were injected with 0.25 μg l-thyroxine/100 g body weight for 2 consecutive days and dosage was increased at 0.25 μg increments, each dose being administered for 2 days with thyroid count made on day of each increase. TSR was determined by plotting per cent previous count at each dosage, and dose which prevented further thyroidal-I^{131} release in each pigeon (95-100% of previous count) was estimated as its TSR.

Results. Rate of release of thyroidal-I^{131} (k′ ′4) was reduced in chickens with reserpine levels of 30 μg or above/100 g body weight (Table I). Body weight losses followed a similar pattern beginning at this level. All birds were quite inactive and decreased food consumption was evident.

Average TSR of pigeons began to decline at levels of 15 μg and above/100 g body weight/day (Table II), but body weight depression also was more pronounced at these levels. At 5 and 10 μg level, TSR was similar to citric acid-injected controls and, correspondingly, body weight loss was not marked. When feed intake was restricted to 66% of controls, TSR

TABLE II. Effect of Varying Levels of Reserpine on Thyroxine Secretion Rate (TSR) and Body Weight in Pigeons.

Reserpine, μg/100 g/day	No. of pigeons	TSR, μg/100 g/day	% change in body wt during reserpine admin.
(Controls)	15	1.26 ± .13*	− 1.1 ± .41
5	16	1.29 ± .12	− 2.0 ± .62
10	18	1.20 ± .16	− 4.2 ± .79
15	15	1.04 ± .09	− 9.3 ± .92†
30	16	.82 ± .02†	−18.3 ±1.02†

* Stand. error. † Significant at $p < .01$.

TABLE III. TSR of Pigeons at Various Levels of Feed Intake.

Treatment	No. of pigeons	TSR, μg/100 g/day	Body wt loss, %
Control (15.5 g/day)	15	1.15 ± .43	5.5 ± 1.0
Group I (66% of control)	18	.85 ± .19	12.0 ± 1.7
Group II (33% of control)	16	.72 ± .12*	15.3 ± 1.9*
Group IV (20% of control)	15	.51 ± .11*	23.0 ± 2.2*

* Significant at $p < .01$.

was depressed 27%, and at 67% and 80% less feed, TSR depression was 37% and 54%, respectively (Table III). At levels of reserpine above 10 μg/day feed consumption and body weight were severely depressed (Table IV). While 60% depression in feed intake and 13.1% body weight loss was observed at 15 μg reserpine level, there was a doubling of body weight loss at 30 μg level (Table IV).

Discussion. Administration of reserpine up to 20 μg/100 g in chickens did not alter release rate of organically bound I^{131}, whereas higher levels produced an inhibitory effect on I^{131} release both in individual and in group comparisons. Coincidentally, loss in body weight followed a similar pattern, *i.e.*, loss occurring only in groups where I^{131} release was also reduced. It was suggestive, therefore, that inanition played more than a passive role in slowing down rate of release of I^{131}. If lower levels of reserpine did reduce thyroid activity, then a certain degree of hypothyroidism would tend to favor fat deposition and cause increase in body weight rather than decrease. Such a situation was never observed. Nevertheless, TSR determination is believed to be most satisfactory index of thyroid function(8). However, observations in pigeons were similar to that in chickens, thyroid inhibition being observed where loss in body weight was observed (Table II) or where feed was restricted (Table III). TSR was reduced only when body weight loss exceeded 9% (Tables II and III). TSR of restricted fed pigeons (Table III) paralleled decreased TSR found at 15 μg/100 g (and above) reserpine level (Table II). Body weight loss and depression in feed intake in pigeons administered graded levels of reserpine (Table IV) paralleled those in whom decreased TSR was noted with increasing levels of reserpine (Table II). When body weight loss was less than 9% and depression in feed intake did not exceed 5%, TSR was not affected, (*i.e.*, when 5 and 10 μg reserpine was administered). No level of reserpine produced thyroid inhibition without depressing feed intake and body weight.

Combined observations in pigeons and chickens indicate that physiological levels of reserpine have no effect on thyroid function in fowls. Thyroid inhibition occurring at relatively high levels of reserpine is very likely due to decreased feed intake. Inanition has been shown to depress thyroid activity(9). Observations of decreased thyroid size and increased cholesterol levels (a measure of inhibition of thyroid function) in serpasil-fed birds with concomitant depression in body weight(10) lends further support to inanition postulation. Observation that reserpine inhibits thyroid activity in some species only indicates species difference. In fowls, physiological levels of reserpine do not have any influence on thyro-pituitary axis.

Summary. Reserpine at levels of 30 μg/100 g/day and above depressed thyroidal-I^{131} release rate in chickens and caused body weight losses. At 15 and 30 μg/100 g/day, thyroxine secretion rate (TSR) was reduced

TABLE IV. Effect of Varying Levels of Reserpine on Body Weight and Feed Intake in Pigeons.

Treatment	No. of pigeons	Depression in feed intake (%)	Body wt loss (%)
Control (citric acid inj.)	18	5.1 ± .98	3.2 ± .82
Group I, 5 μg reserpine/100 g/day	15	6.2 ± 1.0	6.1 ± 1.0
” II, 10 μg ”	16	10.0 ± 1.3	5.5 ± .79
” III, 15 μg ”	15	60.0 ± 5.5*	13.1 ± 1.8 *
” IV, 30 μg ”	16	80.0 ± 6.8*	26.2 ± 3.6 *

* Significant at $p < .01$.

in pigeons with depression in body weight and feed intake. No level of reserpine was found which would inhibit TSR without depressing feed intake. It is suggested that depression of thyroid activity at higher levels of reserpine is secondary to depression in food intake.

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Factor VIII (Antihemophilic Factor) and Factor V (Proaccelerin) Activity of Platelets.* (25819)

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Roskam postulated that normal platelets contain on their surface plasma coagulation factors. He used the expression "plasmatic atmosphere of platelets" to describe this phenomenon(1). Mann *et al.*(2) and later Ware *et al.*(3) showed that normal platelets have factor V activity. Experiments of Hjort *et al.* (4) supported the theory that factor V was adsorbed on the platelet surface. Bounameaux(5) demonstrated the multiplicity of plasma factors adsorbed on platelet surface. The present paper is concerned with 1) demonstrating that normal platelets contain material with factor VIII or factor V activity in quantities sufficient to correct to normal certain diagnostic tests for study of blood from patients with defects of these factors; 2) demonstrating that platelets separated from blood of patients with plasma deficiency of factor VIII or factor V present a defective activity of the corresponding factor; 3) describing some properties of these active factors of platelets.

Materials and methods. Materials and methods used for diagnostic purposes have been described(6,7). All plasma samples used were obtained by mixing blood with 3.8% sodium citrate in proportion of 1:9. Platelets were separated by differential centrifugation and washed 10 times with normal saline. Concentration of normal platelet suspension was then made equal to that of patient's platelets, following a semivolumetric method previously described(6). A 1% suspension v/v was used unless otherwise indicated. When lyophilized platelets were used, weight of dry material was equivalent to that of fresh elements contained in 1% concentration. Platelet suspensions were used in various coagulation mixtures according to technic of "thromboplastin generation test" (TGT), described by Biggs and Douglas(8). Our results are, however, reported in 1 figure (called "prothrombin activation index" or PAI)(6) rather than with a curve. Normal PAI is 9.8 ± 2.9 (2 standard deviations). The systems used are described with specific experiments. Other coagulation mixtures were tested following the basic technic of "prothrombin time" (one-stage method). Systems used are described with specific experiments. Tissue thromboplastin was a commercial preparation obtained from rabbit brain.†

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† Difco Labs., Detroit, Mich.

TABLE I. Factor VIII Activity of Normal Platelets and Absence of Activity of Platelets from 2 Hemophilics and a Patient with Acquired Factor VIII Deficiency. System activated by calcium ions (M/40 $CaCl_2$) and activity estimated on normal substrate.

Plasma	Serum	Platelets	PAI
N	N	N	9.4
H^1	H^1	H^1	38.0
H^2	H^2	H^2	42.1
H^1	H^1	N	11.7
H^1	H^1	H^2	43.2
N	N	H^1	9.6
A	A	A	40.8
A	A	H^1	36.2
A	A	N	12.4

N = Normal. H = Hemophilics. A = Acquired AHF deficiency.

Results. I) *Factor VIII activity of platelets.* 1) Materials for this experiment were Al(OH)-treated plasma diluted 1:5 with normal saline, serum diluted 1:10 with normal saline and platelets. These materials were obtained from a normal subject, 2 hemophilics, and a 62-year-old white man with acquired factor VIII deficiency, and used immediately after preparation. Criteria for diagnosis of classical hemophilia were hemorrhagic diathesis, positive family history, plasma deficiency demonstrable with TGT and absence of correction of plasma defect by mixture with plasma of a known hemophilic. Criteria for diagnosis of "acquired factor VIII deficiency" were severe hemorrhagic diathesis with onset in old age, absence of family history, plasma defect demonstrable with TGT, absence of correction with known hemophilic plasma, abnormal electrophoretic pattern, clinical and laboratory evidence of remissions on steroid therapy associated with correction of electrophoretic pattern to normal. The above materials were variously combined and activity of mixtures estimated on normal substrate. A representative experiment is shown in Table I.

TABLE II. Factor VIII Activity of Platelets Immediately after Separation, after 6 Months of Storage, after Lyophilization and after 24-Hr Incubation at 37°C. Estimation of activity as in Table I.

Plasma	Serum	Platelets	PAI
N	N	N	9.8
H	H	H	42.0
H	H	N O	16.0
H	H	N L	23.0
H I	H I	N	10.9
N	N	N I	18.0

H = Hemophilic. N = Normal. I = Incubated. O = Aged. L = Lyophilized.

2) Materials for this experiment were 6-month-old platelets separated from normal blood and kept in plastic containers suspended in small amount of plasma; platelets lyophilized after separation from normal blood, triple washing with saline and suspension in normal saline. The dry material was kept at room temperature for approximately one year; Al (OH)-treated plasma diluted 1:5 with normal saline; serum diluted 1:10 with normal saline and platelets from normal and hemophilic subjects used immediately after separation; plasmas incubated 24 hours at 37°C, then adsorbed with Al(OH) and diluted 1:5 with normal saline; hemophilic serum incubated at 37°C for 24 hours, then diluted 1:10 with normal saline; normal platelets incubated at 37°C for 24 hours. A representative experiment is shown in Table II.

TABLE III. Factor V Activity of Normal Platelets and Absence of Activity of Platelets from a Patient with Congenital Factor V Deficiency. One-stage method. System activated by tissue thromboplastin and calcium ions (0.02 M $CaCl_2$).

Patient's plasma	Patient's platelets	Normal platelets	Normal saline	Clotting time, sec.
.2				62
.1			.1	70
.1	.1			72
.1		.1		25

II) *Factor V activity of platelets.* 1) Materials in this experiment were plasma and platelets from normal individuals and from a patient with deficiency of factor V. This patient has been described(9). Materials were used immediately after separation. A representative experiment is shown in Table III. 2) Various mixtures were prepared using normal plasma, factor V-deficient plasma, normal platelets and platelets from patient with factor V deficiency. The mixtures were in contact for 18 hours at refrigerator temperature, then platelets were separated by centrifugation and washed 10 times with normal saline. Activity of incubated platelets was estimated by one-stage method using fresh factor V-deficient plasma (obtained from patient mentioned above) as substrate. Results are shown in Table IV.

3) Materials were hemophilic plasma adsorbed with Al(OH) and diluted 1:5 with normal saline either immediately after separation or after 24-hour incubation at 37°C; normal or hemophilic serum diluted 1:10 with saline; platelets separated from normal plasma or factor V-deficient plasma, and used immediately after 10 washings. Hemophilic plasma incubated 24 hours showed "prothrombin time" of 16 seconds and contained less than 5% of factor V activity. Plasma from factor V-deficient patient did not correct this abnormality. The materials were variously combined in TGT systems. Results of representative experiments are shown in Table V.

Comments and conclusions. Serum of hemophilic patients contains larger amounts of prothrombin than normal serum. Elevated serum prothrombin is probably responsible for an apparently smaller deficiency observed when TGT is performed using hemophilic plasma, serum and phospholipidic compounds, as compared with that observed when normal serum is substituted in that system for hemophilic serum. Elevated prothrombin activity of hemophilic serum undoubtedly contributed to reduction of PAI; however, maintaining the system "hemophilic plasma-hemophilic serum" unchanged, values of PAI are much greater when hemophilic platelets are added than when normal platelets are used (Table I). Defective activity of platelets was not characteristic of a single patient nor was it confined to congenital conditions. As expected, hemophilic platelets added to normal plasma and serum showed no abnormality (Table I). It is concluded that activity of platelets is of plasmatic origin. The active factor cannot be completely removed by multiple washings, is stable in stored platelets and in lyophilized material, but appears heat labile (Table II).

TABLE IV. Activity of Platelets from Normal Individual and from Patient with Congenital Factor V Deficiency after Prolonged Contact with Normal Plasma and Factor V-Deficient Plasma. Patient's platelets are devoid of factor V activity but become active after contact with normal plasma. Normal platelets lose their activity after contact with factor V-deficient plasma.

Patient's plasma	Saline	.1 platelet preparation	Clotting time, sec.
.2			67
.1	.1		74
.1		Pp	65
.1		Pn	25
.1		Np	42
.1		Nn	21

N = Normal. P = Factor V-deficient. Small letters indicate source of plasma with which platelets were kept in contact.

TABLE V. Factor VIII Activity of Platelets from a Factor V-Deficient Patient Is Intact. After incubation of hemophilic plasma, factor V was destroyed and platelets from the factor V-deficient patients no longer activate the system. Estimation of activity as in Table I.

Plasma	Serum	Platelets	PAI
N	N	N	9.2
H	H	H	31.2
H	H	N	10.4
H	H	P	13.0
H I	H	N	19.3
H I	H	P	44.6

N = Normal. H = Hemophilic. I = Incubated. P = Factor V-deficient.

Normal platelets demonstrated also factor V activity. Platelets from a patient with factor V deficiency lacked this activity (Table III), but contact with normal plasma resulted in acquisition of activity of previously defective elements. This was not observed when platelets and plasma from the same patient were put in contact (Table IV). Of interest is the loss of some activity by normal platelets when kept in contact with factor V-deficient plasma. This was interpreted as due to consumption of platelet factor V activity during contact, rather than to inhibition. Factor V-deficient platelets corrected a hemophilic mixture almost as well as normal platelets in a hemophilic system, demonstrating that the 2 activities are separable and that factors necessary for evolution of plasma thromboplastin *in vitro* may come interchangeably from platelets or plasma. If both plasma and platelets are deprived of the same factor, thromboplastin formation is defective. This was proved again by heating hemophilic plasma and thus destroying factor V activity; then attempting activation by factor V-deficient platelets. Under these conditions no source of factor V was present in the mixture with decreased thromboplastin concentration in the system (Table V).

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Further Studies on Growth-promoting Factor of Exudates.* (25820)

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Earlier studies indicated that in inflammatory exudates there is present a diffusible growth-promoting factor capable of explaining reasonably the mechanism of repair following an inflammation(1,2). Repeated injections of this factor into breast tissue of non-pregnant rabbits induce lesions closely resembling chronic cystic mastitis(1,2). The cutaneous epithelial structures adjacent to treated nipples likewise display features of marked hyperplasia(2). Furthermore, evidence reported indicated that combination of growth factor of exudates and a carcinogenic hydrocarbon, methylcholanthrene, is followed by further enhancement of proliferative lesions(3,4). The concept has been developed that long standing inflammation may act as a cocarcinogen in presence of a carcinogen, be it exogenous or endogenous(4). The mechanism of action in this important role of inflammation in relation to development of neoplasia appears referable to liberation of growth-promoting factor of exudates(4). Evidence is here presented that activity of the factor appears destroyed by tryptic or ribonuclease digestion. Some of its biological properties are described.

Methods. The growth-promoting factor is present in concentrated diffusate of exudate and is obtained as described earlier(1,2). Chromatographic studies on acid hydrolyzate of the diffusate of exudates indicated association of a peptide with the active principle (2). Spectrophotometric absorption studies have also been conducted with a DU Beckman apparatus. With progress of inflammatory reaction from alkalinity to acidity(5), the diffusate assumes approximately the same pH as the exudate from which it has been obtained. The concentrated diffusate of alkaline exudates is adjusted with N acetic acid to approximately pH 4.5. A small precipitate forms, which augments further after refrigeration. It is then collected by centrifugation at 21,600 x g. This precipitate, washed twice with distilled water, is repeatedly injected into nipples of non-pregnant rabbits by technic previously described(1,2). Untreated nipples as well as nipples repeatedly injected with a buffer at pH 6.2 were likewise studied; the latter to control for the active growth factor, when injected in a medium at acid pH. Irreversibility of effects induced by growth-promoting factor has been tested by periodic observations of nipples for months following cessation of administration of the active principle. Chromatographic studies have been undertaken for presence of nucleotide structures in growth-promoting factor. The material is first hydrolyzed with 70% perchloric acid(6). Descending chromatography is uti-

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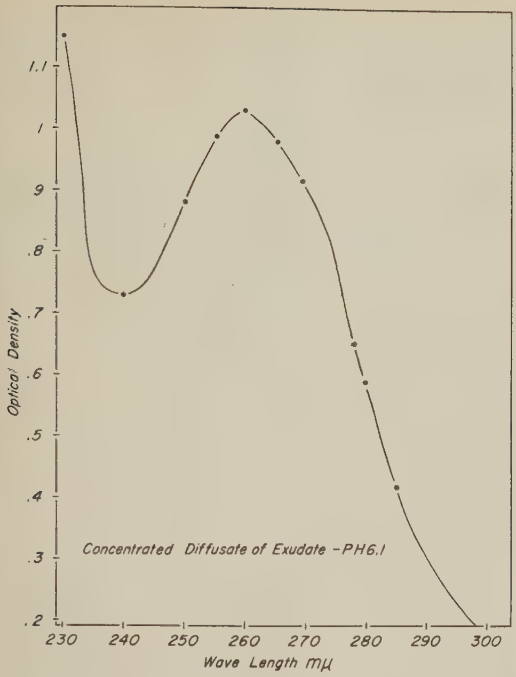


FIG. 1a. Ultra-violet absorption curve from concentrated diffusate of an acid exudate.

lized with 3 types of solvents as follows: 1) N-butanol, ethanol, 5 N HCl, (3:2:2), 2) Aqueous N-butanol (86% by volume), and 3) Isopropyl alcohol 170 ml, conc. HCl 41 ml, H_2O 39 ml(7). The purines, pyrimidines, nucleosides, and nucleotides present are eventually identified on dried Whatman No. 1 paper strips by an ultra-violet lamp. Enzymatic studies with the tryptic digest of growth-promoting factor of exudates have likewise been undertaken to determine whether biological activity is altered. The digest, brought to a boil, to inactivate crystalline trypsin is repeatedly injected into breast tissue of non-pregnant rabbits, and its effect compared on the same animal with that caused by the untreated growth-promoting factor. Ribonuclease hydrolysis of growth-promoting factor has also been undertaken to determine whether nucleotide groups play a part in its activity. These enzymatic studies are properly controlled by simultaneous repeated injections in other nipples of the enzyme *per se*.[†] In some experiments, to avoid injections with ribonuclease, the hydrolyzed ribonuclease growth factor preparation has been dialyzed overnight at 9°C against distilled water.

Results. I. Chemical groups concerned with activity of growth-promoting factor of exudates. The concentrated diffusate derived from acid exudate displays absorption curve with a peak at 260-265 mμ (Fig. 1a). This is consistent with presence of a nucleotide structure in association with the active biological fraction of growth-promoting factor.

The concentrated diffusate of an alkaline exudate is at alkaline pH. The absorption curve tends to flatten between 250 and 280 mμ. The difference between absorption curve of an acid diffusate from an alkaline diffusate is probably referable to a pH effect. As pointed out above, adjusting an alkaline diffusate to acid pH yields a small precipitate,

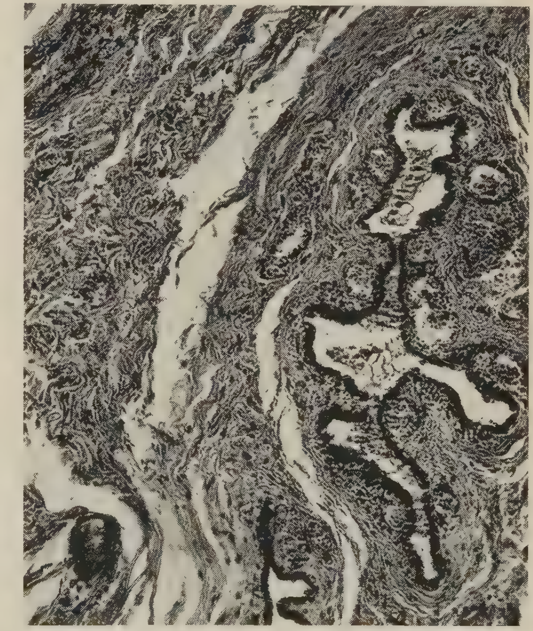


FIG. 1. Rabbit 52-94C. Microscopic picture of breast tissue in 3 wk following 7 injections of concentrated diffusate from canine exudate but previously incubated 1 hr with ribonuclease. Note that ducts and cutaneous epithelium in upper left corner reveal no striking dilatation or hyperplastic changes. Ribonuclease has evidently inactivated the active principle in growth-promoting factor of exudates. × 123.

[†] There is no evidence that ribonuclease utilized contains proteolytic activity. It is a preparation by Nutritional Biochemicals Corp., Cleveland, O.

TABLE I. Effect of Ribonuclease on Inactivation of Growth-Promoting Factor of Exudates.

			Vol of breast tissue underlying nipple (cm³)				
				Nipple inj. with			
Group	No. of inj. of growth-promoting factor with and without exposure to ribonuclease	Duration of exp. (mo)	Untreated nipple	Conc. diffusate	Conc. diffusate but hydrolyzed previously with ribonuclease	Distilled water	Ribonuclease
I Ribonuclease-diffusate dialyzed prior to inj.	24	2	.99	1.45 3.46	1.14 .89	.87	
	28	2.5	.58	2.24 3.02	.16 1.90	.39	
	25	2.5	.23	.55 1.71	.12 .10	.23	
	15	1	.91	1.53 .96	.48 .81		
Avg	23	2	.68	1.87	.70	.50	
II Ribonuclease-diffusate dialyzed for most but not all inj.	31*	3	.30	2.0 2.92	1.16 1.37	.51	
	17*	1.5		1.53 2.12	.85 .65	.23†	
	11*	1	1.16 1.25	5.31 11.21	2.57 6.17	1.42	
Avg	20	1.83	.90	4.18	2.13	.72	
III Ribonuclease-diffusate preparation not dialyzed prior to inj.	25	2	.10	1.56	1.33 .64		.59 .62
	26	2	.24	2.09	1.96 1.34		.35 .31
	17	1.3	1.80	4.21 5.18	3.71	1.46	1.09
	24	2	.20	1.21 1.24	.35	.36	.11
Avg	23	1.83	.59	2.58	1.56	.91	.51

* 6 to 8 injections of ribonuclease-treated diffusate were performed without dialyzing hydrolyzed preparation.

† Received only 9 injections of distilled water.

which in an aqueous medium displays an absorption peak at 260-270 mμ.

Chromatographic studies with 3 different solvents indicate presence of nucleic acid derivatives. Both pyrimidine bases uracil, or its nucleotide, uridylic acid, and cytosine with its derivatives, were most often encountered. Adenine and guanine were also found with all solvents utilized. In brief, the chromatographic data confirm ultra-violet absorption studies, and indicate that a nucleotide appears associated with growth-promoting factor of exudates. Furthermore, ribonuclease tends either to inactivate or reduce the biological activity of the purified growth-promoting factor of exudates (Fig. 1). The results of observations on rabbits on effect of ribonuclease are given in Table I. Observations with ribonuclease hydrolysis were conducted, as a rule, on the same batch of diffusate material as injected in control nipples (Column 5, Table I). This fact rules out the possibility that results obtained are referable to diffusates chemically different in regard, for instance, to phosphorus or nitrogen contents.

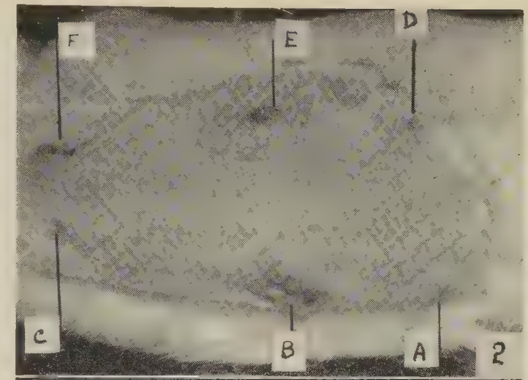
Earlier chromatographic studies demonstrated presence of peptides in association with growth-promoting factor of exudates(2). The Biuret reaction on the diffusate of exudates is, as a rule, negative while the Ninhydrin reaction is positive. The test for presence of SH compounds with Nitroprusside is positive on concentrated diffusate of exudates.

Enzymatic digestion of the factor with trypsin inactivates its biological activity. Six rabbits repeatedly injected for many months into nipple region with tryptic digest of the factor display absence of proliferative effect by either gross or microscopic examination.

In conclusion, inactivation of the growth-promoting factor with either ribonuclease or trypsin suggests that both nucleotide and peptide structures may play an important role in ultimate chemical configuration of growth-promoting factor of exudates.‡

The possibility that a pteridine may also be involved is not consistent with data obtained by spectrophotometry. Pteridines besides having a peak at about 270 mμ reveal a subsequent peak between 320-420 mμ(8). Such a secondary peak was not encountered in measurements conducted on growth-promoting factor of exudates.

II. A. *Irreversible induced effects by proliferative factor.* When administration in breast tissue of growth factor in concentrated diffusate is shortened to about 2 months, and consists of 20 to 25 local injections, upon cessation of injections, the enlarged nipple tends to regress to normal size. When, however, injections are markedly increased and maintained for longer interval, in spite of cessation of injections, a state of irreversibility develops and volume of breast tissue not only fails to regress but it may also progressively increase. The animal shown in Fig. 2 and 3 had received 61 injections of concentrated diffusate of exudates for almost 5 months. Some nipples received also supplementary paintings with 1% methylcholanthrene. The animal was followed for 6 months following cessation of administration of the factor. As seen by comparing nipples B of Fig. 2 and 3, 3 months after discontinuing injections of



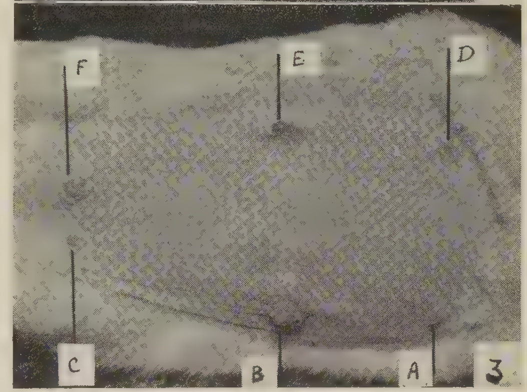


FIG. 2. Rabbit 27-07. For almost 5 mo nipples of this non-pregnant rabbit were treated as follows: A—untreated; B—61 injections of concentrated diffusate of exudate; C—61 injections of physiological saline; D—61 paintings with 1% methylcholanthrene in acetone (first 22 paintings consisted of methylcholanthrene in olive oil, 5% and 1%); E— and F—61 injections of concentrated diffusate of exudate plus 61 paintings with 1% methylcholanthrene in acetone (first 22 paintings consisted of methylcholanthrene in acetone in olive oil, 5% and 1%). At this stage B and E nipples show definite enlargement. Administration of concentrated diffusate or paintings or of both were discontinued, and nipples subsequently studied for irreversible effects (Fig. 3).

FIG. 3. Rabbit 27-07. About 3 mo after cessation of injections of concentrated diffusate of exudate in B area, enlargement of nipple has not regressed, but appears somewhat more prominent than at termination of injections (Fig. 2). Note therefore irreversible effects induced by repeated injections of proliferative factor of exudates. A similar irreversibility of effect seems to have occurred in E nipple in which repeated concentrated diffusate injections and paintings had been administered prior to cessation 3 mo before.

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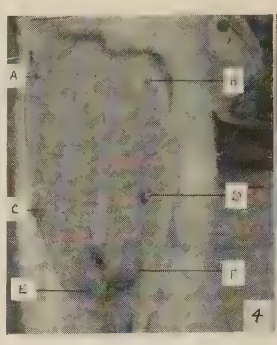
‡ Studies on growth factor of exudates kindly conducted by Dr. Jay Roth of Hahnemann College of Medicine are interesting. He found in 2 determinations by Orcinol reaction, an average equivalent of 0.23 mg/ml of ribose nucleotide and average of 0.27 mg/ml of nitrogen. Since about 12% of nitrogen is utilized in formation of RNA or 0.03 mg nitrogen, there is still 0.27 mg minus 0.03 nitrogen or 0.24 mg nitrogen/ml that can be used in formation of a peptide. It is doubtful that this excess nitrogen is referable to protein since the growth-promoting factor is obtained as diffusible material, and is generally Biuret negative.

the diffusate, the nipple had increased further in size. This significant observation indicates that after passage of a threshold in period and number of injections, a state of irreversibility in growth has been established. This has been observed in several rabbits and will again be described in connection with injections of a further purified fraction obtained from concentrated alkaline diffusate material by adjusting it to pH 4.3-4.6 with N acetic acid. Microscopically, months after cessation of injections, hyperplastic effects in cutaneous epithelium has also failed to regress appreciably. Furthermore, breast tissue of injected nipples, several months after cessation of injections, manifests considerable interductal fibrosis and cystic dilatation of the ducts. This is not seen in either untreated nipple or in nipple repeatedly injected with physiological saline.

B. *Thermostability of growth factor.* The factor appears heat stable. Several non-pregnant rabbits repeatedly injected in their nipples with concentrated diffusate of exudate, and also with the same material, but first brought to a boil and cooled, display no significant difference in end results. The factor therefore appears to be relatively heat stable. This is not wholly surprising since both nucleotides and polypeptides are known to be heat stable.



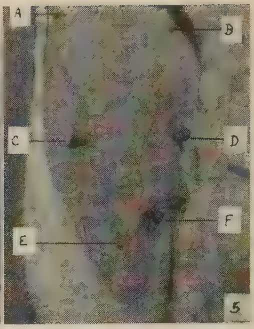
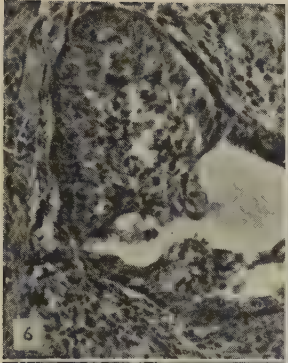


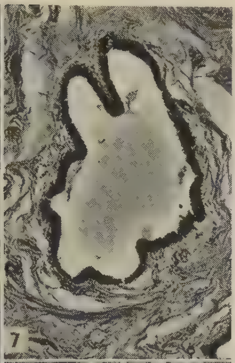
FIG. 4. Rabbit 19. Effect of repeated injections of purified fraction of growth factor of exudates on breast tissue of non-pregnant rabbit. Nipples A and B untreated. Nipples C and D inj. 34 times each with purified fraction of growth-promoting factor for 3 mo. Photograph taken 2 wk after cessation of injections. Note that nipples (particularly D) are enlarged. Nipple F inj. 21 times with crude concentrated diffusate of exudate for 2 mo. In this nipple there developed a superimposed suppurative inflammation. Nipple E inj. 13 times with phosphate buffer at pH 6.2 for one mo induced no appreciable effect.

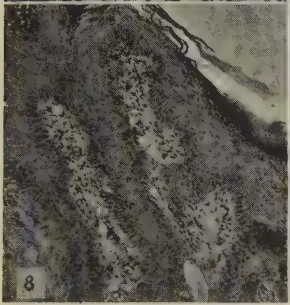
FIG. 5. Rabbit 19. Effect of repeated injections of purified growth-promoting factor of exudates on breast tissue of non-pregnant rabbit. Nipples A and B untreated. Nipples C and D inj. 34 times each with purified growth factor for 3 mo. Three and a half mo following cessation of injections, enlarged nipples C and D failed to regress, but have enlarged further (*cf.* Fig. 4). Nipple F inj. 21 times with crude concentrated diffusate material from exudates for 2 mo. There developed also a superimposed inflammatory reaction with suppuration. In nipple E, 13 injections of phosphate buffer at pH 6.2 for one mo induced no appreciable effect.

C. *Biological effects of active fraction recovered as growth-promoting factor of exudates.* The growth-promoting factor of exudates can be recovered as a precipitate at about pH 4.5 by adjusting the concentrated diffusate with N acetic acid. This precipitated material at times brought back to pH 7.0 with diluted NaOH, when injected into nipples of rabbits, induces marked proliferative effects and enlargement of nipples (Fig. 4, nipples C and D) (following 34 administrations for 3 months). The photograph was taken 2 weeks after ceasing administration of active growth material. Three and a half months after termination of injections the nipples not only had not regressed, but had enlarged further (Fig. 5). The growth process had evidently reached irreversibility. In nipple E (Fig. 5), 13 injections had been performed each with 0.5 ml of phosphate buffer at pH 6.2 as a further control. Repeated injections of the buffer at acid pH had no appreciable growth effect on the nipple (Fig. 5). Similar observations were made on 8 non-pregnant rabbits repeatedly injected into breast tissue with this active growth-promoting fraction of exudates.

Microscopic examination of breast tissue of a nipple repeatedly injected with this further purified fraction of growth factor of exudates reveals marked proliferative activity of the lining epithelium in the ducts (Fig. 6) (following 22 injections of the active fraction for 7 weeks). Note pseudostratification and squamous metaplasia as epithelial cells protrude into lumen of the duct. There is some, but no appreciable infiltration of chronic inflammatory cells at periphery of the duct (Fig. 6). Appearance of duct resembles chronic cystic







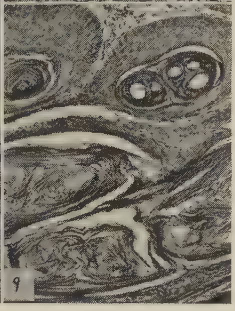


FIG. 6. Rabbit 38-91D. Appearance of a duct in breast tissue of nipple inj. 22 times with purified growth-promoting fraction of exudates over 7 wk. Note marked proliferative effect of epithelium as it protrudes into lumen. This recalls that encountered in chronic cystic mastitis of human beings. × 104.

FIG. 7. Rabbit 38-91A. Appearance of ducts in untreated breast tissue taken from same animal as in Fig. 6. Epithelial lining of ducts is normal in contrast to Fig. 6. × 75.

FIG. 8. Rabbit 38-91. Appearance of cutaneous epithelium adjacent to nipple inj. 22 times within 7 wk with purified fraction of growth-promoting factor of exudates. Note marked hyperplasia with dipping down of "tongues" of epithelial tissue containing also mitotic figures. Specificity of effect of growth factor of exudates is indicated by its absence in concentrated blood serum diffusate when latter is extracted by same procedure. Repeated injections of physiological saline also fail to induce similar proliferative effects.

FIG. 9. Rabbit 38-91. Appearance of hair follicles adjacent to nipple inj. 22 times in 7 wk with purified fraction of growth-promoting factor of exudates. Note marked hyperplasia and considerable keratinization. × 75.

mastitis of humans. This is in marked contrast to appearance of untreated breast in the same animal (Fig. 7). The ducts fail to reveal evidence of hyperplasia in the lining epithelium. Cutaneous epithelium in the vicinity of repeatedly injected nipple displays also marked epithelial hyperplasia with "tongues" of this tissue projecting deep into the corium (Fig. 8). In this actively proliferating epithelium, mitotic figures are found in moderate numbers. Adjacent hair follicles likewise reveal considerable hyperplastic effects. Keratinization is found in abundance in numerous hair follicles (Fig. 9).

Discussion. Enzymatic observations reported here indicate that growth-promoting factor of inflammatory exudates contains peptide and nucleotide structures concerned in its activity. Diffusibility, thermostability, ultra-violet absorption, and chromatographic studies of this factor are consistent with this view. Hydrolysis with trypsin inactivates biological activity of growth factor of exudates. Hydrolysis with ribonuclease likewise appreciably inactivates it. Growth factor of exudates in its further purified state displays striking proliferative effects both on breast tissue and on adjacent cutaneous epithelial structures.

The presence of this factor in exudates offers a reasonable explanation for the mechanism of repair following inflammatory reaction. It is conceivable that the factor in its present state or in a more purified form may have practical application in wound healing. This aspect is being studied. Continuous chemical studies to determine the precise nature of growth-promoting factor of exudates are desirable. The possibility of its being a nucleopeptide is merely suggestive, and is being studied. Growth factor of exudates is specific in causing proliferative responses, for when blood serum is substituted in the extracting procedure the final fraction displays no proliferative effectiveness(1,2,9). As shown earlier, repeated utilization of an acute inflammatory irritant such as croton oil in blood serum fails to induce proliferative effects(9). Repeated saline injections likewise yield no proliferation(2). When, however, a protracted chronic inflammation leads to a proliferative state, it is conceivable that growth-promoting factor is liberated by injured cells at the site of inflammation.

Isolation of a growth factor from inflammatory exudates may also aid in further elucidation of the role of inflammation in carcinogenesis. The evidence indicates that repeated administration of the factor produces not only progressive growth of breast tissue in non-pregnant rabbits, but also, after 30 to 35 injections irreversibility occurs whereby the enlarged nipple not only fails to regress in size, but increases, though administration of growth factor has been discontinued (*cf.* Fig. 3, 4, 5, 6). It is unlikely, however, that growth factor *per se* can induce a cancer. It is more likely, as pointed out elsewhere, that it acts as a cocarcinogen in accelerating the effect of a carcinogen, whether of exogenous or of endogenous origin(4). The latter may in turn be liberated as a result of somatic mutation or genetic susceptibility. This may be the real significance of the growth factor of exudates in evaluating the precise role of a long standing inflammation in carcinogenesis.

This growth factor may help to clarify how a long standing inflammatory response sometimes gradually develops into a neoplasm (1,2). It is possible that the diverse ways in which neoplasms can be induced may be in part explained in terms of a final common pathway, and this pathway may be through liberation of a growth-promoting substance by mildly injured cells(1,2,3,4,9). This type of injury may be the result of chronic irritation, virus infection, or hormonal imbalance(9). However, there is little doubt that the growth factor released by injured cells is in itself insufficient to induce development of a neoplasm. Initiation of the process by some unknown genetic mechanism seems of primary importance. Liberation by a variety of means of the endogenous growth factor from injured cells seems only to act as a further promoter or cocarcinogen to a more fundamental process.

Summary. There is present in inflammatory exudates a diffusible growth-promoting factor which can be purified further from concentrated diffusate by precipitation at pH 4.5 with N acetic acid. Repeated injections of the fraction in its present state of purification induce in non-pregnant rabbits conspicuous hyperplasia of epithelial structures as well as a condition in breast tissue simulating that seen in chronic cystic mastitis. A state of irreversibility is established upon termination of a prolonged course of injections of growth-promoting factor of exudates. The enlarged nipples fail to regress in size, though administration of the active material is discontinued. By enzymatic inactivation, evidence is advanced that both nucleotide and peptide structures appear to play a role in biological activity of the growth-promoting factor of exudates.

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Alteration in Intestinal Microbial Flora of Rats with Tail Cups to Prevent Coprophagy. (25821)

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There is indirect evidence from studies of animals fed diets containing sulfonamides or antibiotics that intestinal microorganisms synthesize vitamins of importance to host. This concept has been strengthened lately by the fact that germfree rats develop a severe Vit. K deficiency on a Vit. K-free diet whereas conventional animals on the same diet do not show any symptoms(1). Signs of folic acid deficiency have been found in germfree rats on a deficient diet, but animals with normal microbial flora on the same diet show no signs of folic acid deficiency(2). However, the question still remains whether the vitamins synthesized in intestinal tract of laboratory animals are utilized by direct absorption or only after coprophagy. Barnes and Fiala(3) have demonstrated that rats showed signs of Vit. K deficiency when coprophagy was prevented by means of a plastic tail cup. Control rats on the same Vit. K-free diet had no symptoms. These findings suggest that the vitamins synthesized by the microbial flora of rats are utilized mainly through recirculation of the feces. The possibility exists, however, that use of the tail cups produces qualitative or quantitative changes in the microbial flora thus diminishing synthesis of the vitamin. One way of investigating this possibility is to evaluate intestinal flora of animals with tail cups through direct bacterial counts. The aim of the present investigation has therefore been to compare intestinal microbial floras in groups of rats with and without tail cups. The lactobacilli were chosen for study because they constitute an important sector of the microbial flora of intestinal tracts of rodents(4, 5), and because a satisfactory medium was available for their selective isolation and enumeration(6,7).

Materials and methods. Two series of rats were used in the study. The animals of *Series A* were part of a study on influence of a pyridoxine deficient diet on frequency of urinary calculi in rats with and without tail cups. In *Series A* there were 4 groups of weanling Sprague-Dawley rats. Group No. 1 had a nondeficient diet without tail cups; group 2 had the same diet and tail cups. Groups No. 3 and 4 had a pyridoxine-deficient diet without and with tail cups respectively. Numbers of lactobacilli were determined on the cecal contents of these animals at the end of the experimental period of 8 weeks. In *Series B,* 36 rats of both sexes from the Wistar strain used at Notre Dame Univ. for germfree research were weaned on the 21st day to a diet low in Vit. K(1). Bacterial counts of the fresh feces were made on the same day making up the baseline value for both groups. At 22 days of age tail cups were put on half of the rats and lactobacillus counts of the feces were then recorded once a week in both groups to the age of 9 weeks when all rats were sacrificed. Numbers of lactobacilli were then also determined in the contents of the ceca. All animals were kept in individual cages with screen bottoms. Food and water were available *ad libitum.* For lactobacillus counts, the SL medium of Rogosa(6) was modified by reducing glucose content to 1.0% and adding 0.5% L-arabinose and 0.5% sucrose. Weighed amounts of fresh feces or cecal contents were serially diluted in 0.03% sterile yeast extract water and plates were poured at final dilutions of 10^2, 10^4, and 10^6. In *Series B* plates were also poured at a dilution of 10^8. Plates were counted after 4 days incubation in air at 37°C. The only organisms other than lactobacilli found to grow on the SL medium in the present study were occasional colonies of filamentous yeasts of the genera *Geotrichum* or *Candida.* These were easily distinguished by their large size and

TABLE I. Lactobacillus Counts of Cecum Contents in *Series A*.

Group	Pyridoxine	Tail cups	No. of animals	Mean No. of lactobacilli/g*	Distribution of animals according to No. lactobacilli/g*		
					$<10^2$	10^2-10^6	$>10^6$
1	+	—	12	$2.8 \cdot 10^7$	0	2	10
2	+	+	9	$4.5 \cdot 10^6$	1	4	4
3	—	—	11	$1.6 \cdot 10^8$	0	0	11
4	—	+	11	$3.1 \cdot 10^7$	2	6	3
1, 3	+ and —	—	23	$9.1 \cdot 10^7$	0	2	21
2, 4	+ and —	+	20	$1.9 \cdot 10^7$	3	10	7
1, 2	+	+ and —	21	$1.8 \cdot 10^7$	1	6	14
3, 4	—	+ and —	22	$9.5 \cdot 10^7$	2	6	14

* Based on wet wt.

typical growth on the surface of the medium. The statistical analyses were performed by the Chi square method.

Results. Series A. There was a significant difference in distribution of lactobacillus counts in cecal contents of the rats with and without tail cups in Series A (Tables I, II). This difference was also present when comparison was made between the cupped and the noncupped rats on the pyridoxine containing diet. Pyridoxine deficiency resulted in a marked growth retardation but had no statistically significant influence on number of lactobacilli, although mean values seem to be higher in the deficient groups.

When the means of number of bacteria for noncupped and cupped rats are compared, the former have about 5 times more lactobacilli than the latter. The value of comparison of the means of bacterial counts is debatable, however, in view of the disproportionate influence of single high or low values. For instance, in the group of rats on the pyridoxine free diet and without tail cups there were 2 rats with such high values that counting was impossible even in the highest dilution.

TABLE II. Statistical Analysis of Distribution of Lactobacillus Counts in *Series A*.

Pyridoxine	Tail cups	Probability
+ —	— —	p >.5
+ —	+ +	”
+ +	+ —	.01 > p >.001
— —	+ —	p <.001
+ and — + and —	+ —	”
+ —	+ and — + and —	p >.5

Series B. At the start of the study and the day before the tail cups were applied, there was no difference between the 2 study groups in lactobacillus counts of the feces (Table III and Fig. 1). During the following 3 weeks the groups with tail cups showed a considerable decrease in lactobacilli. This decrease is statistically significant both by *intra-* group and *inter*-group comparison. The decrease amounts to about 5 log values in the means by *intra*-group and 3 log values by *inter*-group comparison. This lower difference by *inter*-group comparison in turn depends on a down-

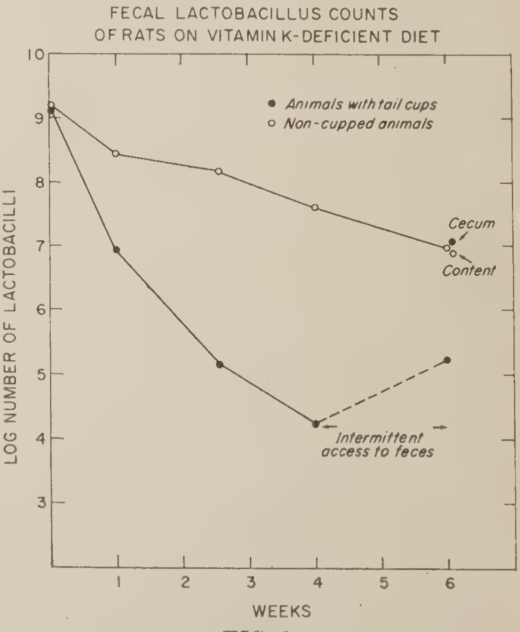

FIG. 1.

TABLE III. Lactobacillus Counts in Series B.

Time in wk after cupping	Material	Tail cups	No. of animals	Mean No. of lactobacilli/g*	Distribution of animals according to No. of lactobacilli/g*			Probability
					$<10^2$	10^2-10^6	$>10^6$	
0	Feces	+	18	$1.1 \cdot 10^9$	0	2	16	$p > .5$
		—	"	$1.6 \cdot 10^9$	0	0	18	
1	"	+	"	$9.5 \cdot 10^6$	0	10	8	$.05 > p > .01$
		—	"	$4.6 \cdot 10^8$	0	4	14	
2.5	"	+	"	$1.7 \cdot 10^5$	1	16	1	$p < .001$
		—	"	$1.8 \cdot 10^8$	0	7	11	
4	"	+	"	$2.6 \cdot 10^4$	2	16	0	"
		—	"	$5.9 \cdot 10^7$	0	9	9	
6	"	+	"	$2.5 \cdot 10^5$	0	17	1	"
		—	17†	$9.9 \cdot 10^6$	1	8	8	$.01 > p > .001$
6	Cecum content	+	18	$1.6 \cdot 10^7$	0	14	4	$p > .5$
		—	17	$8.6 \cdot 10^6$	0	11	6	

* Based on wet wt; initial counts performed before cups were applied.

† One animal died of pneumonia. No sign of hemorrhage.

ward trend also in the rats without tail cups. When initial values of this latter group are compared with those in the same group at 3 weeks, this downward trend is just on the borderline of being significant ($0.10 > p > 0.05$).

Prior to 5th week, the animals never had access to their feces whereas during the 5th and 6th weeks almost all were able to practice coprophagy for at least one day due to faulty operation of the tail cups. Eight of the animals succeeded at least once in freeing themselves completely of the cups. Of these 8 rats all had higher numbers of lactobacilli than during the preceding week. In 3 cases the increase was more than 2 log values. Among the remaining 10 animals in this group, 7 showed an increase while the counts of 3 animals declined. The fact that all the rats with the tail cups could practice coprophagy to a certain extent during the last 2 weeks could also be an explanation for the lack of difference between cupped and noncupped rats as far as lactobacillus counts of the cecal contents are concerned. Another possibility is that the difference between counts in the feces of rats with and without tail cups is due to bacterial population changes in the tail cups after feces have been voided. If this were so, however, it is difficult to understand why lactobacillus counts increased in the feces of the cupped rats when these rats were able to practice coprophagy.

The present study demonstrates the importance of coprophagy for maintenance of the intestinal flora. Although these observations have been confined to the changes in the population of intestinal lactobacilli, it is obvious that the balance between other types of microorganisms in the intestinal environment might also be affected when coprophagy is prevented. Consequently in the study of specific vitamin deficiencies in animals with tail cups, one must consider not only the factor of reduced intake of the vitamins usually to be found in the feces but also the possibility of variations in intestinal levels of vitamins due to synthesis or depletion by the altered intestinal microflora.

Summary. Under conditions in which coprophagy was completely prevented by use of tail cups, the number of lactobacilli in cecal contents and feces of rats was reduced significantly. Thus, the possibility exists that studies of vitamin deficiencies observed under conditions of cupping may be complicated by changes in intestinal microflora.

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Adrenal Gland Weights of Hereford and Brahman Cattle. (25822)

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Information about weights of organs may be helpful in estimating functional status of the organ. This concept has been widely used with endocrine glands since some methods of assaying hormone output have been based upon gland weight. Hartman and Brownell (1), Bachmann(2) and Chester Jones(3) have recently reviewed the scattered data concerning adrenal weights of the rat, man, dog, rabbit, cat and mouse, but little or no information is presented on cattle adrenals. Sisson and Grollman(4) merely describe the shape of the adrenal glands and their normal position in the bovine body, without mentioning size or internal anatomy. In view of the paucity of information concerning weight of adrenals of cattle, the following data are presented.

Methods. Adrenal glands were obtained from 119 Hereford (*Bos taurus*) and Brahman (*Bos indicus*) beef cattle, slaughtered by standard abattoir procedures. Fifty-eight were steers, 5 were bulls and the remainder females. All animals except bulls were slaughter cattle, selected at random from groups of known Herefords or Brahmans. All animals had carcass grade either U.S. Standard or Good. Animals were transported to slaughterhouse holding pens on day previous to killing and given access to water only. On day of slaughter animals were weighed, then stunned, bled, skinned and dressed. To avoid damage to adrenal glands, they were removed before the carcass was split. This removal occurred approximately 15 minutes after death. Adrenal glands were cleaned of excess fat and the gland from each side of animal weighed separately. One gland taken at random from each of 13 Hereford heifers was dissected into cortical and medullary regions under dissecting microscope, then weighed.

Results. Fresh adrenal and body weights for each group of animals are presented in Table I. Data of weight ratios for the 2 groups of steers and Hereford heifers were subjected to analysis of variance since each group was sufficiently large and comparable in size. The Hereford heifers had a significantly larger ($P<.05$) adrenal to body weight ratio than either group of steers, while there was no significant difference in weight ratios of the 2 steer groups. Weights of cortex and medulla for the 13 dissected glands are presented in Table II.

Discussion. Adrenals of beef cattle are much smaller in relation to body weight than those of most other mammals examined. Expressed in mg, adrenal weight/100 g of body weight (mg %) the male rat, according to Korenchevsky(5), has a ratio varying from 42 to 14 decreasing with age, and the human decreases from over 100 g/100 kg body weight at birth to 30 g at puberty and 25 g at 70 years of age. Other instances of higher ratios of adrenal weight to body weight are cited by Chester Jones(3). Published data on adrenal weights of cattle are summarized in Table III, and would appear to confirm the low gland to body weight ratios observed in cattle by the authors. The data for the Angus and Jersey cows(6) differ from the remainder possibly because of the very high

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TABLE I. Mean Adrenal Weights for the Various Groups of Cattle.

Cattle data			No. of animals	Mean live wt, kg	Mean adrenal wt, g			Adrenal, live wt ratio, g/100 kg
Breed	Sex	Age, yr			Pair	Left	Right	
Hereford	St.	1½	30	403	19.0	9.6	9.4	4.46 ± .66
Brahman	St.	1½	28	373	16.2	8.2	8.0	4.34 ± .80
Hereford	♀	3+	29	362	19.1	10.1	9.0	5.16 ± 1.36
Brahman	♀	3+	10	404	14.3	7.9	6.4	4.53 ± 1.84
"	♀	¾	17	156	6.8	3.4	3.4	4.36 ± 1.11
Hereford	♂	3+	3	581	29.6			5.13
Brahman	♂	3+	1	472	23.9	12.2	11.7	5.06
"	♂	¾	1	173	7.7	3.7	4.0	4.45

TABLE II. Weights of Adrenal Cortex and Medulla.

No. and group	Mean of single adrenal wt, g	Mean wt of adrenal, g		% of adrenal wt	
		Cortex	Medulla	Cortex	Medulla
13 Hereford heifers	6.6 ± 1.57	4.5 ± .79	2.1 ± .36	69.	31.

condition of these animals, both of which were show ring champions receiving special attention for many years.

In rats, adrenal weight to body weight ratio declines with age(5), as it does in children up to puberty(3). In this study with cattle an opposite effect is observed with adrenal weights slightly increasing with age. This trend is confirmed by the observations of others (Table III).

Adrenal weight in many mammals appears to vary with estrus, pregnancy, difference in strain and body weight(3). The females in this study were not in estrus on the day of slaughter nor were any pregnant, and since the animals slaughtered came at random from many strains, these variables cannot be discussed in relation to the presented data. In this study the adrenal weight to live weight ratio increased slightly with age and weight. This effect might have been even more marked if weight ratios were calculated on a true body weight (fat free) basis, since much of the weight increase in beef cattle is extracellular fat.

Data in Table I show that Hereford cattle in all groups had a slightly larger adrenal to live weight ratio than the Brahmans. The species differences were examined statistically and found to be not significant, however. A similar trend was demonstrated in other data(8) where the Santa Gertrudis and

TABLE III. Adrenal Weight (g) to Live Weight (100 kg) Ratio for Cattle as Calculated from Data from Other Sources.

Data source	Breed	Management	No.	Mean live wt, kg	Mean adr. wt, g	Adr. wt/Live wt
Swett *et al.*(6)	Angus	Beef	1	720	57.5	7.99
(7)	Hereford	"	3	374	19.9	5.32
Adult ♀ (7)	"	Dairy	4	370	17.4	4.70
(7)	Holstein	"	2	627	30.3	4.83
(6)	Jersey	"	1	421	37.0	8.79
Black(8)	Angus	Beef	2	393	16.47	4.19
1-yr-old steers	Hereford	"	6	374	16.08	4.30 ± .17
	Santa Gertrudis	"	7	431	15.90	3.69 ± .30
	Brangus	"	9	394	15.01	3.81 ± .65
Tucker(9) 1-yr-old steers	Crossbred	Beef	21	297	12.40	4.18 ± .76
Bedrak(10) 2-yr-old heifers	Hereford	Beef	18	325	13.57	4.18 ± .64
Brody & Kibler(11) Adult ♀	Mixed	Dairy		488	31.00	6.35

Brangus, 2 breeds with some Brahman blood, had relatively smaller adrenals per unit of live weight.

In most mammals examined to date, weight of the left adrenal exceeds weight of the right (2,3). The data on bovine adrenal gland weights presented here agree for the most part with this finding. The significance of these differences is probably limited to differences in anatomical makeup of each species.

Although there is evidence that the adrenals of women, female mice and female rats are larger than those of the males, many species do not exhibit this sex difference(3). Beef cattle apparently are in the latter category. In species which exhibit a sex difference in adrenal weight, castration results in an increase in adrenal cortex, particularly the zona fasciculata(3). This cortical change is brought about by a change in the balance of gonadotrophins and steroids. Although the gonads had been removed from the steers several months prior to slaughter, no increase in adrenal weight was detected, but rather there was a slight decline in the adrenal body weight ratio. Data from other sources(8,9) would tend to confirm the ratio decline following castration, which may be real or could be apparent due to increase in extracellular fat which is not an increase in true body weight.

Adrenal medulla in adults of higher mammals does not change in size very much or very quickly, while the cortex is prone to rapid changes in size(3). In female mice the cortex represents approximately 82% of the whole gland whereas in the male mouse the cortex makes up about 70% of the whole adrenal. In rats and humans the cortex accounts for about 90% and in dogs some 82% of the entire gland. Although limited, data in this study suggest that female Herefords possess an adrenal gland with a much smaller cortex than is reported for other species. The cortex of some of the adrenals taken from Hereford heifers were histologically examined by Bedrak(10). The mean width in millimeters of the various cortical zones for 20 Hereford heifers as determined microscopically were capsule, 0.217; glomerulosa, 0.501; fasciculata, 1.269; and reticularis 0.701. The zona fasciculata represented about half the cortex in these cattle, which is much less than that for man(3).

Summary. Weights of individual and paired adrenals together with adrenal-body weight ratio for male, female and castrated Hereford and Brahman cattle are presented. There was no significant difference between weight ratios for the 2 species although adrenal-body weight ratio was consistently smaller for Brahman cattle. Unlike most other mammalian species examined, adrenal-body weight ratio tends to increase slightly with age. Adrenal weights of cattle are apparently not affected by sex. Castration decreased adrenal-body weight ratio, which is the reverse of that observed in other species. While the left adrenal was consistently larger than the right, total adrenal weight was much smaller in relation to unit body weight than any other mammalian species for which data are available. The bovine adrenal cortex appears to be smaller in relation to the whole gland than for most other mammals as does the zona fasciculata.

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Correlation of Arachidonic Acid of Serum Cholesterol Esters in Different Species with Susceptibility to Atherosclerosis.* (25823)

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Data on serum cholesterol ester fatty acid (CEFA) composition of different species are limited. Cow and human plasma cholesterol esters have been shown(1-6) to contain a large proportion of linoleic acid (40-50% of total CEFA), while arachidonic acid accounts for only 5-10%. The CEFA of dog plasma (7) contain a much higher proportion (up to 25%) of arachidonic acid than that of man, but in this species linoleic acid also predominates. Rat blood cholesterol esters have been reported(8) to consist principally of saturated fatty acids, while in another study(9) oleic acid was found to be the major CEFA.† Sinclair(10) has recently hypothesized that a deficiency of arachidonic acid, or the factors necessary for arachidonic acid synthesis (linoleic acid and pyridoxine), may be important in development of atherosclerosis in man. Recent studies(6,11) from these laboratories with the aid of gas-liquid chromatography have shown that arachidonic acid constitutes up to 50% of serum CEFA of the rat and only 7.5% in serum CEFA of man. The correlation between arachidonic acid of the CEFA of those 2 species and their well known differences in susceptibility to development of atherosclerosis suggested that determination of the CEFA of other species would be of interest.

Methods and materials. All animals used in this study were males, with one exception noted in Table I. The animals (5-6 per group) were as follows: rats (Wistar strain, 250-350 g), guinea pigs (Hartley strain, 300-350 g), chickens (White Leghorn, 1-2 years), geese (mixed breeds, 1-2 years), pigs (Yorkshire and Hampshire, 1-2 years), rabbits (albino, 6 months), dogs (mongrels, 1 year and older). The diets of the different species and their fatty acid composition are indicated in Table III. The animals were fasted overnight prior to collection of blood samples. Blood was obtained from 6 fasted human subjects, aged 61-74 years, in good nutrition, on the usual hospital diet (30-40% fat). Lipid extracts of individual serum samples and diets were prepared as described earlier(12). Free and total cholesterol of each extract were determined by the method of Sperry and Webb (13). Cholesterol esters were separated from the other lipid components by chromatography on silicic acid(14). The isolated cholesterol esters were interesterified in HCl-methanol and methyl esters were sublimed according to procedure of Stoffel *et al.*(15). Gas-liquid chromatography was carried out as previously described(6).

Results. Serum CEFA compositions of the different species are shown in Table I. The rat had the highest content (50% of arachidonic acid in the serum cholesterol ester fraction followed by the dog with 17%. The remaining species (man, chicken, goose, rabbit, pig and guinea pig) had much smaller amounts of arachidonic acid in the cholesterol ester fraction. Linoleic acid was the major acid in dog, man, rabbit, guinea pig and pig, with the guinea pig having the highest percentage (57.9%) of that acid. The goose and chicken had substantial amounts of linoleic acid (approximately 30%), but oleic acid was the major acid in the cholesterol esters of those species. Also, there were differences in distribution of saturated and other monoenoic acids in the cholesterol ester fractions characteristic of the different species.

Table II shows serum cholesterol levels of the different species. Man and goose had the highest average total cholesterol level

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* This study supported by grants from PHS and Am. Heart Assn.

† While this paper was in preparation, Howton, D. R., and Hashimoto, S., *Am. J. Clin. Nutrition*, 1960, v8, 50, also reported high levels of arachidonic acid (67.6%) in rat plasma cholesterol esters.

TABLE I. Serum Cholesterol Ester Fatty Acid Composition of Different Species.

Fatty acid* Chain length carbons	No. double bonds	Rat (5)†	Dog (5)	Man (6)	Goose (6)	Chicken (6)	Rabbit (6)	Pig (5)	Guinea pig (6)
		% total fatty acids							
6 to 12		1.7 ± .4‡	.7 ± .3	1.0 ± .2	.8 ± .1	.4 ± .2	.7 ± .2	.8 ± .1	2.8 ± .5
14	0	.8 ± .2	.6 ± .4	.9 ± .2	.3 ± .1	.3 ± .1	.8 ± .2	.5 ± .1	1.1 ± .5
”	1	.5 ± .3	.2 ± .1	.3 ± .1	tr	tr	.6 ± .2	.2 ± .0	.6 ± .4
16	0	12.4 ±2.1	12.2 ±1.9	13.7 ±1.4	17.2 ±1.5	15.1 ±2.2	21.3 ±2.0	12.8 ±2.0	18.6 ±3.4
”	1	2.4 ±1.1	2.9 ±1.0	4.1 ±1.1	1.7 ± .6	6.9 ±1.5	2.1 ± .6	2.9 ± .7	2.4 ± .6
”	2	tr	tr	.4 ± .1	tr	tr	tr	.2 ± .0	tr
18	0	2.6 ± .3	3.2 ±1.1	1.3 ± .3	2.4 ± .3	6.1 ±1.9	3.5 ±1.2	4.2 ± .9	.5 ± .2
”	1	9.8 ± .9	18.1 ±1.6	25.8 ±3.5	40.0 ±8.1	35.8 ±8.9	24.6 ±3.4	30.8 ±3.7	14.8 ±2.5
”	2	19.5 ±1.9	44.9 ±6.3	43.3 ±4.7	31.3 ±7.7	29.3 ±7.1	43.0 ±3.2	45.4 ±2.5	57.9 ±3.1
”	3	.3 ± .3	tr	.7 ± .4	.3 ± .2	.3 ± .1	1.1 ± .9	.4 ± .2	.2 ± .2
20	0	tr	tr	.7 ± .4	.2 ± .1	.2 ± .1	tr	tr	tr
”	3	tr	.2 ± .1	.3 ± .2	tr	.4 ± .2	tr	tr	tr
”	4	50.0 ± .9	17.0 ±6.5	7.5 ±2.2	5.8 ± .3	5.2 ±2.1	2.3 ± .5	1.8 ± .8	1.1 ± .7

* Represents major fatty acids found; small amounts of others were detected. † Values in parentheses represent No. of animals; animals were males with exception of 3 geese. ‡ Stand. dev.

(227 and 228 mg respectively). The dog had an intermediate total serum cholesterol level of approximately 130 mg% and the remaining species had levels less than 100 mg%. In all species studied, percent of esterified cholesterol in total serum cholesterol was in the narrow range of from 69.2 to 79.0%.

Diets of the different species (Table III), except man, were low in fat (2.4-4.2%). Appreciable amounts of linoleic acid (29.3-54% of total dietary fatty acids) were present in fat of all diets. The diets of the rat, rabbit and guinea pig contained approximately 1% of linoleic acid. That of the chicken and goose contained 1.7%. The remainder of the dietary fatty acids were principally palmitic and oleic acids.

Discussion. The serum CEFA pattern is different for each species and does not appear to be related to dietary intake of fatty acids. Those species (goose and chicken) which consumed a diet high in linoleic acid had low percentages of linoleic and arachidonic acids in their serum cholesterol ester fractions, whereas the CEFA of the rat, which consumed a diet containing less linoleic acid, had the greatest amount of polyunsaturated fatty acids. It would appear that those animals with high CEFA arachidonic acid levels (rat and dog) can more easily utilize their linoleic acid for arachidonic acid production or may incorporate more of the available arachidonic acid into cholesterol esters.

Data in Table I indicate that there is a correlation between susceptibility to atherosclerosis of the different species and content of arachidonic acid in cholesterol esters of their sera. The rat is extremely resistant to production of atherosclerosis by artificial means(16,17). The dog is also highly resistant by the usual cholesterol feeding technic, although the disease can be produced if thiouracil is added to a high cholesterol diet(18). In the dog arachidonic acid in CEFA, while high, is considerably lower than in the cholesterol ester fraction of the rat. In the goose, chicken, rabbit, guinea pig and pig atherosclerosis has been readily produced by feeding of cholesterol(19-23). In those species the atheromatous lesion bears a marked simi-

TABLE II. Serum Cholesterol Levels of Different Species.

Species*	Serum cholesterol, mg %			Ester/Total, %
	Free	Ester	Total	
Rat	20.6 ± 1.8†	41.5 ± 13.5	70.1 ± 13.0	69.9 ± 5.6
Dog	31.1 ± 8.9	98.3 ± 37.5	129.4 ± 45.3	76.0 ± 4.7
Man	60.0 ± 9.9	166.5 ± 36.9	226.5 ± 46.5	73.5 ± 1.6
Goose	70.4 ±20.2	158.0 ± 30.9	228.4 ± 38.0	69.2 ± 7.4
Chicken	33.7 ± 9.8	119.2 ± 35.9	152.9 ± 45.2	78.0 ± 2.0
Rabbit	11.8 ± 3.3	27.4 ± 10.3	39.2 ± 12.6	69.9 ± 5.5
Guinea pig	10.8 ± 2.3	32.3 ± 7.7	43.1 ± 9.5	74.9 ± 3.2
Pig	14.7 ± 3.6	55.4 ± 9.9	70.1 ± 12.4	79.0 ± 3.3

* Represents avg of 5 to 6 animals. † Stand. dev.

larity to that of man. All of those species were found to have low arachidonic acid levels in their serum cholesterol esters(1.1-5.8%). Man is susceptible to atherosclerosis, but seems to be somewhat more resistant than the other species (arachidonic acid 7.5%). The disease takes many years to progress to important lesions in man, while only a short high-cholesterol feeding period is necessary for its production in the susceptible species. It is also of interest that spontaneous atherosclerosis(24-27) has been observed in goose, chicken, rabbit and swine. The dog develops atherosclerosis spontaneously, but lipid appears to occur to a minimal extent or is absent from the lesion(28). No definite correlation between levels of fatty acids, other than arachidonic, in the CEFA and susceptibility to atherosclerosis is apparent. Also, there is no correlation between serum cholesterol level and species susceptibility to atherosclerosis.

TABLE III. Dietary Fatty Acid Composition and Fat Content.

Fatty acid		Diets of species*			
Chain length carbons	No. double bonds	Rat	Dog	Chicken, goose	Rabbit, guinea pig
		% total fatty acids			
6 to 12		.3	.4	tr	.3
14	0	1.6	1.0	tr	.6
”	1	.4	tr	tr	tr
16	0	23.6	28.1	13.7	18.3
”	1	2.0	1.6	.2	.9
18	0	8.5	7.1	2.3	2.5
”	1	32.1	26.1	28.9	23.4
”	2	29.3	33.2	54.0	48.6
”	3	2.2	2.5	.9	5.4
Dietary fat content,† %		4.2	2.5	3.1	2.4
Linoleic acid content,‡ %		1.0	.7	1.7	1.1

* Represents analyses of Purina rat chow, Purina rabbit and guinea pig chow, cracked corn (chickens and geese), and Purina dog chow.

† Fat content of diets consumed by the pigs was unavailable.

‡ Calculated as % of diet.

Our results are in accord with Sinclair's hypothesis(10) that a deficiency of arachidonic acid may be an important factor in etiology of atherosclerosis. These findings are evidence only by association. It remains to be seen whether levels of arachidonic acid in the tissues, perhaps particularly in fatty acids of cholesterol esters, may be increased and whether that increase in arachidonic acid may reduce susceptibility to atherosclerosis. The low content of fat in the diets of species other than man suggests that differences in proportion of arachidonic acid in the CEFA may be a characteristic of the species which will not be readily affected. The large amounts of saturated, monoenoic and short-chain fatty acids and the uncertainty of the adequacy of pyridoxine content in the modern diet of "western" man leaves open the possibility of increasing arachidonic acid content of his tissues.

Summary. Serum CEFA composition of 8 different species has been determined by gas-liquid chromatography. Different CEFA spectrums were found for each species. The outstanding variation was the proportions of arachidonic acid in CEFA of the serums. That of the rat had 50% of arachidonic acid and that of the dog had 17%. All species also had a large proportion of linoleic acid in the CEFA of their serums. The rat and the dog, with high arachidonic acid levels in CEFA of their serums, are known to be highly resistant to development of atherosclerosis.

Those species having small proportions of arachidonic acid in the CEFA of their serums are susceptible to atherosclerosis and develop the disease spontaneously. This study provides evidence, by association, that level of arachidonic acid in the CEFA may be related to susceptibility to atherosclerosis. It remains open to investigation whether the proportion of arachidonic acid in the CEFA is a characteristic of the species or whether it, and the susceptibility to atherosclerosis, can be altered by dietary measures.

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Association between Presence of Transplanted Leukemia and Morphological Changes in Hosts' Thyroid Glands.* (25824)

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We observed that presence of spontaneous mammary adenocarcinoma in C3H parous females is associated with decreased epithelial height and increased diameter of thyroid follicles as compared to non-tumor-bearing controls(1). These changes suggested that presence of tumor is associated with decreased thyroid function of host(2). This investigation was initiated to determine if this phenomenon might be more widespread in its occurrence, as suggested by earlier observations of Larinow(3) that mice with various types

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TABLE I. Thyroid Measurements,* Body Weight, and Spleen Weight in Leukemic and Non-Leukemic C57 Male Mice.

Group	No. mice	Mean body wt (g)	Mean spleen wt (mg)	Mean epithelial ht	Mean diameter
Control—no leukemia	29	20.0	102.9	7.27	46.99
C1498 present, 8 days	12	22.2	123.8	7.04	47.81
" " , 11 "	17	23.6	226.8	5.97	48.07

* In μ.

of cancer show a depression in height of follicular epithelium.

Methods. Inbred C57 Bl/6/JAX male mice were housed in transparent plastic cages and fed Purina Laboratory Chow *ad lib.* with oat supplement. Temperature was controlled between 78-80°F. Two-month-old animals were inoculated with fragments of myelogenous leukemia C1498‡ subcutaneously in the right axillary region by a No. 12 trocar. Non-inoculated littermates served as controls. Thyroid glands were handled and examined as previously reported(1). The mean of 10 measurements from the thyroid of each animal was recorded and the mean for each experimental group calculated. Spleen weights were recorded as indication of extent of leukemic involvement.

Results. Eight days following leukemia inoculation, thyroids of leukemic mice showed lower epithelia and larger follicular diameters than those of non-inoculated controls (Table I). Since these differences were rather small and not statistically significant, a second series of mice similarly treated was sacrificed 11 days after inoculation. After the neoplasm had been in host for 11 days, considerable proliferation had occurred, as indicated by increase in spleen weight over that observed at 8 days. At 11 days the difference in mean epithelial height of control and leukemic group was statistically significant. Mean total body weights of leukemia-bearing mice in both series were greater than those for control mice. Only a portion of this increased body weight is accounted for by splenomegaly observed in leukemic animals. Neither massive lymphatic involvement nor ascites was apparent and hepatomegaly, although it may have been present, was not massive and would not account for a significant proportion of increased body weight. Mice in both series appeared alert and healthy. Experiments extending beyond 11 days were not feasible due to ensuing poor condition and high mortality of leukemia-bearing animals.

Discussion. The results suggest that there is a lowered thyroid function in presence of leukemia C1498 in C57 Black mice. Similar observations in C3H female mice bearing spontaneous mammary tumors(1) and in mice with spontaneous, transplanted and induced tumors(3) indicate that this phenomenon is not limited to strain of mouse or type of neoplasm employed. Related observations on tumor-bearing rats indicate that this phenomenon is non-species specific(4).

Stevens *et al.* noted that in rats(5) and mice(6) there is a lower concentration of injected I^{131} in thyroids of tumor-bearing than in tumor-free animals, thus strengthening the view that our morphological observations of thyroids are a reflection of functional activity.

These observations may be related to the "iodine-trapping syndrome" described in tumor-bearing rats and mice by Scott *et al.*(7). This syndrome is characterized by a higher than normal retention of I^{131} by skin, muscle, gastrointestinal tract and plasma and lower uptake by thyroid and low excretion in urine. The relationship of these observations to elevated metabolic rates in patients with either myeloid or lymphoid leukemia is not clear since such elevated rates may or may not be accompanied by changes in thyroid function (8,9).

Summary. C57 male mice which had been inoculated 11 days previously with leukemia C1498 had statistically significantly lower thyroid follicle epithelium than did untreated controls. Thyroids of inoculated mice also had slightly, though not statistically significantly, larger follicle diameters. These changes in thyroid morphology were present but less extensive 8 days after tumor inoculation.

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‡ Obtained from R. B. Jackson Memorial Lab.

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Adrenocortical and Gonadal Responses of Female Mice to Increased Population Density. (25825)

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Increased adrenal cortical and decreased gonadal function result from grouping male mice, the magnitude of these responses depending on population density(1). Similar gonadal and adrenocortical changes occur in female mice from populations of mixed sex (2,3,4,5). The changes in male mice are related to intraspecific strife, their magnitude in an individual to its position in a social hierarchy(6). It is not known if the adrenals of female mice and rats react similarly when in groups of females only, although it is known that reproduction is adversely affected with suppression of estrus(7,8). This paper explores the effects on adrenal glands and gonadal function of placing females together in groups in absence of males.

Methods. In each of 3 experiments female mice were placed in individual cages at 12-13 g. Four weeks later the mice were randomized and assigned according to groups or to remain isolated. Ten days after grouping, the mice were sacrificed and weights of adrenals, thymus, spleen, ovaries, uterus and, in Exp. 3, the preputials obtained after fixation in 10% neutral formalin(2,9,10). Adrenals were serially sectioned at 6 μ in all experiments, and ovaries, uterus, and vagina were sectioned in Exp. 3. Widths of adrenal X-zones, zonae fasciculata-reticularis, and number of cells in straight cords of the latter were determined histologically(2). Eleven groups of 6 each of nulliparous females of the albino NMRI strain were used in Exp. 1. Seven groups of 6 parous mice each, of the same strain, were used in Exp. 2. Parous mice were used to avoid confounding the adrenal weight data by the X-zone, which is involuted at first pregnancy in females. For this experiment male and female littermates were placed, one pair to a cage, at weaning. Males were removed when the females were obviously pregnant for the first time, at 10 or 11 weeks of age. The young were removed at birth and the females isolated until the required number were obtained. Seven groups of 4 each of inbred, nulliparous females, of a strain originally derived from wild mice, were used in Exp. 3. This strain is much more aggressive and more reactive to grouping than laboratory albino mice(9). It is known that females of this strain are in estrus a high percentage of the time and do not undergo spontaneous pseudopregnancy.

Results. The results are summarized in Table I as mean organ weight and its stand-

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TABLE I. Organ Weights Expressed as Means and Standard Errors of Nulliparous Albino (Exp. 1), Parous Albino (Exp. 2), and Nulliparous Wild Stock (Exp. 3) Female Mice, in Groups Consisting of Females Only, and with Their Isolated Controls. In grouped mice, the means are means and standard errors of group means.

Exp.		No.	Mean body wt change	Adrenals	Width X-zone	Width fasc.-ret.	Cells fasc.	Thymus	Spleen	Ovaries	Uterus	Preputials
1	Isolated	12	24.3 ± 1.0	9.70 ±.78	15.9 ± 2.9	16.5 ±.5	15.6 ±.6	74.6 ± 5.1	107.5 ± 6.7	10.2 ± .8	104.7 ± 14.6	
	Grouped	11	26.0 ± 1.9	10.1 ±.41	11.1 ± .8	20.3 ±.3	21.4 ±.7	87.0 ± 2.4	117.4 ± 5.3	11.3 ± .4	94.5 ± 19.3	
	p		.5	.70	.02	.0001	.0001	.06	.50	.70	.70	
2	Isolated	16	− .8	7.83 ±.38	5.5 ± 1.3	20.8 ±.8	19.0 ±.7	54.9 ± 3.9	127.7 ± 5.4	16.1 ±1.1	158.8 ± 14.1	
	Grouped	7	−3.9	7.60 ±.30	2.8 ± .6	23.8 ±.6	21.6 ±.8	60.4 ±10.3	145.9 ± 7.2	17.2 ±2.9	177.1 ± 6.7	
	p			.70	.10	.01	.05	.70	.06	.50	.30	
3	Isolated	7	+2.7 ± .3	12.08 ±.53	17.1 ± 2.0	17.5 ±.5	16.9 ±.6	74.3 ± 6.1	86.3 ± 9.6	7.71 ± .69–	156.7 ± 15.8	13.1 ± 2.5
	Grouped	7	+3.7 ± .3	11.66 ±.31	17.6 ± .8	21.1 ±.8	18.9 ±.4	65.5 ± 4.0	99.8 ± 8.5	9.14 ± .40	75.1 ± 4.1	15.1 ± 2.0
	p		.05	.50	.90	.01	.02	.30	.40	.10	.001	.30

ard error. Only mean values for each group are used for purposes of statistical evaluation, to avoid within-cage effects and to test reliably the effects of grouping. Consequently, mean weights given for the grouped animals are means of the group means.

There is no significant decline in X-zone width in the grouped mice in Exp. 3. However, histologically the X-zones of grouped mice showed marked involutional changes not seen in their isolated controls. Also, uterine and vaginal sections from this experiment indicated that most of the grouped mice were in anestrus or diestrus at sacrifice, whereas 5 of the 7 isolated controls were in estrus. Uterine weight appeared closely related to stage of the estrous cycle. Lower uterine weight in grouped mice reflects the fact that most were diestrus or anestrus, conversely with the isolated controls. If the stages of estrus are assigned values as follows: anestrus O, diestrus 1, metestrus 2, proestrus 3, and estrus 4, the grouped mice have a mean "estrus value" of 1.27 ± 0.24 and the isolated mice 3.29 ± 0.47, the difference being highly significant ($P<0.01$).

Discussion. Grouping appears to result in an increase of adrenocortical activity in female mice, which is not reflected by adrenal weight since mild hyperplasia of the adrenal fasciculata-reticularis in grouped females was accompanied by an equivalently increased involution of the X-zone. Combined widths of the fasciculata-reticularis and X-zones remained constant. The X-zone is involuted by weak androgens(9,11), some of which are known to be secreted by the adrenal cortex (12-15) and to be stimulated by ACTH(13-16). Increased involution of the X-zone of grouped mice may have been caused by increased adrenal androgens. Greater mean preputial weight in grouped mice (Exp. 3), although not significant, is consistent with this interpretation and increased ACTH is indicated by hyperplasia of the fasciculata-reticularis. The marked adrenocortical response in female rats and mice from populations of mixed sex must largely be due to the presence of males, increased female aggressiveness during pregnancy and lactation, or both.

Splenic weight in male mice increases with grouping(17,18). Mean splenic weight of grouped females, in every experiment, was greater than in isolated mice, although the differences were not significant. It seems logical to conclude that grouping induces an increase in splenic size in female mice.

Ovarian weight was not affected by grouping. Uterine weight was greater in isolated than in grouped nulliparous wild stock and albino nulliparous female mice, but not significantly in the latter. However, since the changes in the albinos were in the same direction as the significant change in wild stock mice, it appears that a diminution in uterine size is associated with grouping, which is consistent with the results of Andervont(7) and of Whitten(8), showing inhibition of reproductive function in grouped female mice. The fact that decreased uterine weight correlated with diestrus and anestrus in the wild stock mice and that this strain is known not to undergo spontaneous pseudopregnancy lends weight to the conclusions. Isolated wild stock females are in estrus a major portion of the time, so that a large proportion in diestrus, with infantile uterine weights, suggests that these mice were anestrus, corresponding to Whitten's(8) results.

Summary. Nulliparous and parous albino, and nulliparous wild stock female mice were subjected to grouping. Weights of adrenals, thymus, spleen, ovaries, and uterus were obtained. Widths of the adrenocortical X- and fasciculata-reticularis zones were measured and number of cells in columns of fasciculata-reticularis determined. Weights of adrenals, thymus, and ovaries were not significantly affected by grouping. Increased adrenocortical activity with grouping was indicated by cellular hyperplasia of the zonae fasciculata-reticularis. Increased involution of the adrenal X-zone with grouping may be accounted for by increased adrenal androgens. Diminished uterine weight in grouped nulliparous females suggests suppression of gonadal function and gonadotrophin secretion. Based partly on prior evidence, it was concluded that an increase in splenic weight was associated with grouping. The observed responses account only in small part for the degree of adrenocortical activity and reproductive suppression in populations of mixed sex.

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Serum Insulin-Like Activity as Measured by Mouse Hemidiaphragm Technic. (25826)

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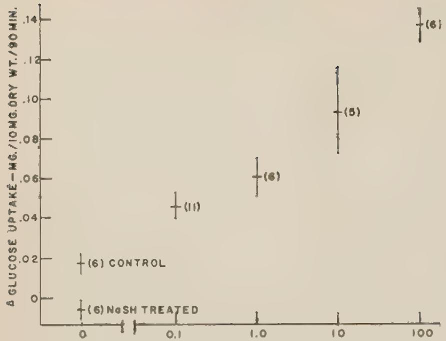
We described(1) a method for estimating minute amounts of insulin based on its stimulating effect on glucose uptake by isolated mouse diaphragm tissue. In the present study we have applied this technic to serum using stimulation of glucose uptake as an index of "serum insulin" or more precisely of serum insulin-like activity.

Method and materials. The general procedure for preparing diaphragms and measuring glucose uptake previously described(1) was employed except for the following modifications: (a) number of hemi-diaphragms in each pool was reduced from 5 to 4, (b) Mg-free standard Krebs - Ringer - bicarbonate (KRB) solution(2) was employed in incubation medium in place of the previously used high bicarbonate containing modified KRB solution, (c) diaphragms were not chilled during preparation as was done previously but rinsed in buffer solution kept at room temperature. The test incubation medium consisted of 2.0 ml of KRB-glucose (KRBG) solution and 1.0 ml of KRB-dialyzed serum sample. Glucose was dissolved in the KRB solution (1.5 mg/ml) so that initial glucose concentration of the incubation medium was 100 mg%. The control medium was identical to the test medium except for substitution of KRB solution (glucose free) for the serum. Serum insulin-like activity is expressed as net increase in glucose (mg) disappearing from the test medium/10 mg dry wt of diaphragm tissue/90 min incubation over its corresponding control. *Serum samples:* Within 3 hours after a blood sample was taken, serum was separated and dialyzed for a minimum of 3 days against daily changes of fresh KRB solution stirred by a magnetic stirrer in the cold. Blood was obtained by cardiac puncture from Osborne-Mendel rats and Swiss-Webster mice anesthetized with sodium pentobarbital (30 mg/kg). Dog, human, and rabbit blood were obtained without anesthesia. Alloxan-diabetes was produced by injecting alloxan intravenously: 70 mg/kg for rats and 200 mg/kg for rabbits. Rats were maintained by subcutaneous administration of 1 to 2 units of protamine zinc insulin 6 days a week; rabbits were given 4 units. Animals were used no earlier than 2 months following alloxan treatment. Animals were fasted for 24 hours and insulin administration discontinued 48 hours prior to bleeding and only those animals having a blood sugar value over 300 mg% were used in these experiments.

Results. Fig. 1 shows activity of normal rabbit serum to which increasing amounts of insulin were added and then dialyzed according to test procedure. The control to which no insulin was added indicates the endogenous insulin-like activity initially present in serum. Treatment of control serum with $NaSH \cdot 2H_2O$ (25 mg/ml) for 30 minutes at room temperature completely eliminated this activity. Although as little as 0.1 milliunit insulin/ml of serum increased significantly glucose uptake over control serum, comparison of responses to insulin in this serum to that in buffer medium alone (Fig. 2) indicated a possible inhibitory action of normal serum on exogenous insulin.

Other serum protein solutions were studied for their effect on insulin activity (Table I). In these experiments 1 milliunit of insulin in 0.1 ml volume was added to the test incubation medium which consisted of 1.0 ml of the dialyzed protein solution and 2.0 ml of KRBG solution. In each case the corresponding control medium was identical in composition with the test medium except for omission of insulin. Differences in endogenous insulin activity of the various protein solutions were corrected by this procedure. Also, by direct addition of insulin to incubation medium the possibility of loss during the dialysis procedure was eliminated. All protein solutions used had a relatively low endogenous activity, less



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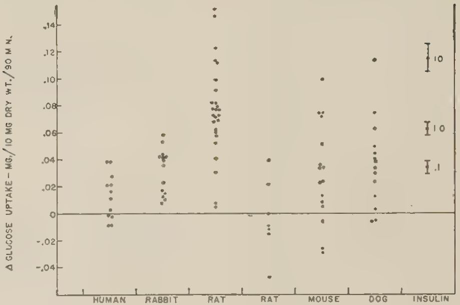

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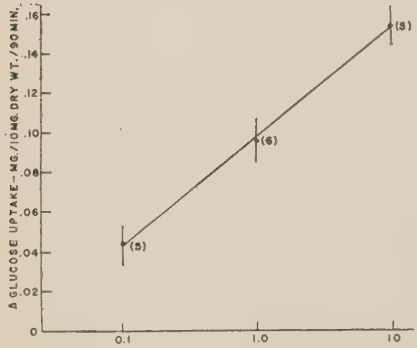

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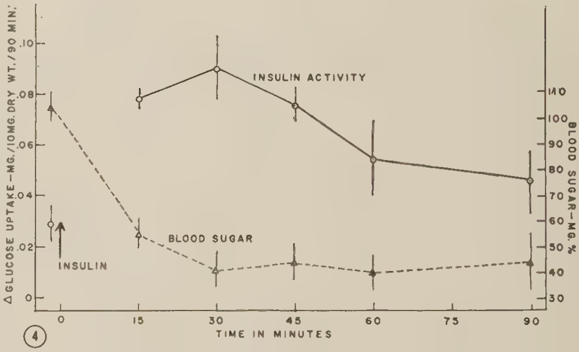

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FIG. 1. Mean effect of insulin added to normal rabbit serum. No. of experiments in parentheses; stand. errors as vertical bars.

FIG. 2. Log dose—response of insulin in KRB buffer medium. Milliunits insulin in 3.1/ml total incubation vol.

FIG. 3. Comparison of serum insulin-like activity of various species. See text.

FIG. 4. Serum insulin activity and blood glucose values in rabbits following intrav. inj. of crystalline zinc-insulin, 1 unit/kg. Mean ± stand. error from 5 animals.

than 0.02. Maximum activity of 0.133 effected by 1 milliunit insulin was obtained in the beef serum albumin (BSA) medium (1.6% final conc.). In support of the previously observed inhibitory action of normal serum, activity of insulin in the presence of normal rabbit serum was significantly less than in the KRB medium alone. Although insulin activity measured in the presence of diabetic rabbit serum appeared to be greater than in normal serum, the difference was not statistically significant. Ability of normal rabbit serum to inhibit insulin response was unaffected by diluting the serum as much as 10-fold; ac-

TABLE I. Effect of Serum Proteins on Response to Insulin.

Medium		Glucose uptake, mg/10 mg dry wt/90 min. Control	1 milliunit insulin	Effect	P
(A) Beef serum albumin 1.6%	(9)	.401 ± .007	.535 ± .011	.133 ± .009	A–B <0.025 A–C <0.01 A–D <0.001
(B) Buffer only	(6)	.450 ± .014	.547 ± .013	.096 ± .011	B–C <0.5 B–D <0.05
(C) Rabbit serum, diabetic	(4)	.378 ± .037	.461 ± .032	.084 ± .008	C–D <0.2
(D) Rabbit serum, normal	(8)	.453 ± .018	.517 ± .023	.064 ± .009	

No. of experiments in parentheses. Glucose uptake values = mean ± stand. error. P = level of significance between indicated media.

tivity of 1 milliunit insulin was 0.060 ± .01 in undiluted serum and 0.062 ± .008 in serum diluted 10-fold.

Fig. 3 shows serum insulin-like activity of various species as measured by mouse diaphragm technic. Serum samples were from non-fasted dogs and humans; rabbits, rats, and mice were fasted for 24 hours. Mean activity ± S.E. for each species were: human (0.013 ± .005), rabbit (0.031 ± .004), rat (0.075 ± .007), alloxan-diabetic rat (0.003 ± .010), mouse (0.030 ± .009), and dog (0.036 ± .009). Insulin concentrations (milliunits/ml) corresponding to these mean activities are: human (0.02) rabbit (0.09), rat (1.8), alloxan-diabetic rat (0.0), mouse (0.08), and dog (0.13). These values were calculated from a response curve of insulin standards measured in presence of rabbit serum (Fig. 3, last column). Insulin response from 6 replicate analyses was: 0.114 ± .011 for 10 milliunits, 0.062 ± .005 for 1 milliunit, and 0.033 ± .005 for 0.1 milliunit. Mean activity of normal rats was significantly higher than in any other species studied. Of 7 samples tested from alloxan-diabetic rats, 5 showed no insulin-like activity. Suppression of glucose uptake by a sample of diabetic rat serum below the uptake of its corresponding buffer control appeared to be related to degree of lipemia since the most opaque sample showed the most inhibitory action. In initial attempts to measure insulin-like activity of mouse serum (6 mice/pool), blood was obtained by decapitation. Serum obtained by this procedure consistently showed no significant activity and added insulin (2 milliunits/ml) was not recoverable. These observations may be explained by release from damaged tissue cells of insulin degrading factors(3,4) into blood obtained by decapitation.

Fig. 4 shows mean serum activity and blood sugar level of 5 rabbits before and after intravenous injection of insulin (1 unit/kg). Insulin was injected into the marginal ear vein of one ear and blood samples taken from the opposite ear vein at the indicated times. A significant increase ($p<.005$) in activity from the pre-injection level was noted in all rabbits at 15, 30 and 45 minutes; calculated insulin concentration at these times were 2.2, 3.5, and 1.9 milliunits/ml respectively compared to 0.08 milliunit/ml for initial serum concentration. At 60 and 90 minutes the concentration was calculated to be 0.55 and 0.27 milliunit/ml but these levels were not significantly different from initial pre-injection level.

Discussion. Although most workers employing the diaphragm technic for estimating "serum insulin" use undialyzed serum or plasma, we have employed dialyzed serum because: a) it obviates tedious adjustments in amount of glucose added to incubation medium to compensate for variations in glucose concentration of test serum samples; b) it eliminates dialyzable serum constituents which may possibly affect glucose uptake and; c) it minimizes possible changes effected by serum on pH and buffering capacity of incubation medium which have a marked influence on glucose uptake(1). Dialyzed serum samples have been kept at 4°C for as long as 2 weeks without measurable loss of insulin activity.

Serum insulin concentration is usually calculated by comparison of serum response directly with response of insulin standards which are run in a medium devoid of normal serum proteins. In view of the inhibitory effect which we have found of normal whole serum on insulin response, it is evident that calculations based on such a comparison will give lower values for "serum insulin" than responses corrected for this inhibitory effect of serum. We have attempted to correct for this inhibitory effect of serum by determining response of insulin reference standards in presence of normal serum (rabbit).

Studies are in progress to determine the nature and degree of this inhibitory effect of normal serum: whether it varies from individual to individual and whether there are species differences. Accurate transformation of net glucose uptake effect to equivalent insulin concentration can be made only when this is known. It is apparent that the inhibitory activity probably resides in the non-albumin fractions of whole serum since BSA did not inhibit. The actual enhancing effect of BSA on insulin response compared to that obtained in buffer medium alone may be due to its protective action in preventing loss of insulin by adsorption or degradation.

Comparison of "serum insulin" levels in normal animals have shown that there is a considerable range among normal individual animals; normal rats, for example, have shown as much as a 1000-fold variation in serum activity. Although some of this scatter may be a reflection of the inherent variability of the bioassay technic employed, results so far lend support to the view that significant changes of "serum insulin" occur under normal physiologic conditions. The most interesting observation in comparing relative activities in the various animal species was the marked difference between human and rat serum insulin-like activity. The order of increasing activity found was: human$<$mouse, rabbit, dog$<<$rat. Because of its significantly higher "serum insulin" level, rat serum should be particularly useful in studies to characterize the serum constituent which stimulates glucose uptake and to establish its relationship with "serum insulin."

The general shape of the insulin clearance curve in rabbits (Fig. 4) is similar to that obtained by Scott and co-workers(5) who employed a higher dose (15 units/kg) than the 1 unit/kg dose used in our studies. Although they found a 6-fold maximum increase in insulin concentration over pre-injection level, we have found over a 40-fold increase while employing 1/15 the amount of injected insulin. This difference may be explained in part of the much higher value of 2.5 milliunits/ml they report for normal rabbits before injection compared to 0.08 milliunit/ml which we have found using the mouse diaphragm technic.

Summary. The mouse diaphragm technic has been adapted to measure insulin-like activity of dialyzed serum. Compared to the response effected by insulin in KRB buffer medium alone, normal rabbit serum inhibited while beef serum albumin enhanced insulin response. Mean serum insulin level of adult rats was significantly greater than rabbits, mice, and dogs and much higher than humans. A significant elevation in serum insulin activity which persisted up to 45 minutes following injection, was found in rabbits injected intravenously with 1 unit/kg insulin.

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Immune Electron Microscopy. (25827)

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In the past decade advances in the study of immune reactions of bacterial, viral, and mycotic agents have been made, using immunofluorescent technics. It has become apparent that even more refined technics are desirable because of the low resolution with this technic, to prove that in some systems the reactions are specific and not due to nonspecific uptake of the fluorescent dye, and also to provide a means of studying pathogenesis at a subcellular level. Singer(1) prepared an electron-dense conjugate by coupling ferritin to an antibody, indicating that specificity of the antibody was probably not altered. Using his concept, an immune serum to *Staphylococcus aureus* was conjugated to ferritin by a slightly modified method, to serve as a model for study of immune reactions at the subcellular level.

Materials and methods. To 10 ml (0.5 g) of horse spleen ferritin was added 20 ml of 0.5 M carbonate-bicarbonate buffer, pH 9.0, at 4°C. With slow stirring, to prevent frothing, 0.6 ml of m-xylylene diisocyanate was added dropwise and stirring was continued for three-quarters of an hour at 4°C. The supernatant, the ferritin conjugate (FeIso), was removed by centrifugation and was allowed to stand in the cold for 1 hour. An immune serum to *Staphylococcus aureus*, Type I, (ATCC 12598) was prepared from a boiled broth culture according to Hobbs(2). The serum was diluted with physiologic saline to contain 10 mg protein/ml, 10% of total volume being the carbonate buffer. At 4°C an equal volume of FeIso conjugate was added dropwise with slow stirring, and the stirring continued for 2 days. The mixture was dialyzed in the cold for 5 hours against 0.1 M ammonium carbonate and overnight against 0.1 M phosphate buffer, pH 7.2. A clear, brownish conjugate was obtained after centrifugation. The preimmunization serum was similarly treated to serve as a control. An 18-hour agar culture of the homologous organism was fixed in 10% buffered formalin and washed in buffered saline. The suspension was divided into 2 aliquots. One aliquot was allowed to react with the immune conjugate, the other with the control conjugate. Both were washed, run through graded alcohols, and embedded in methacrylate with uranium(3). Thin sections were cut and placed on a grid and observed with the RCA EMU 3D electron microscope with a 1 mil objective aperture at initial magnification of 9,000 for Fig. 1 and 2. Fig. 2 insert was taken at an initial magnification of 30,000.

Results. The ferritin-antibody conjugate retained its specificity, and antigen-antibody reaction was easily detected by the characteristic pattern of the ferritin molecule. The ferritin was completely washed out of the background, preventing confusion with a specific reaction. The control (Fig. 1) shows the capsule-like material as being a homogeneous entity, while the test with immune serum (Fig. 2) shows ferritin molecules in the capsule-like material, indicating an antigen-antibody reaction. Fig. 2 (insert) shows the molecular structure of ferritin as described by

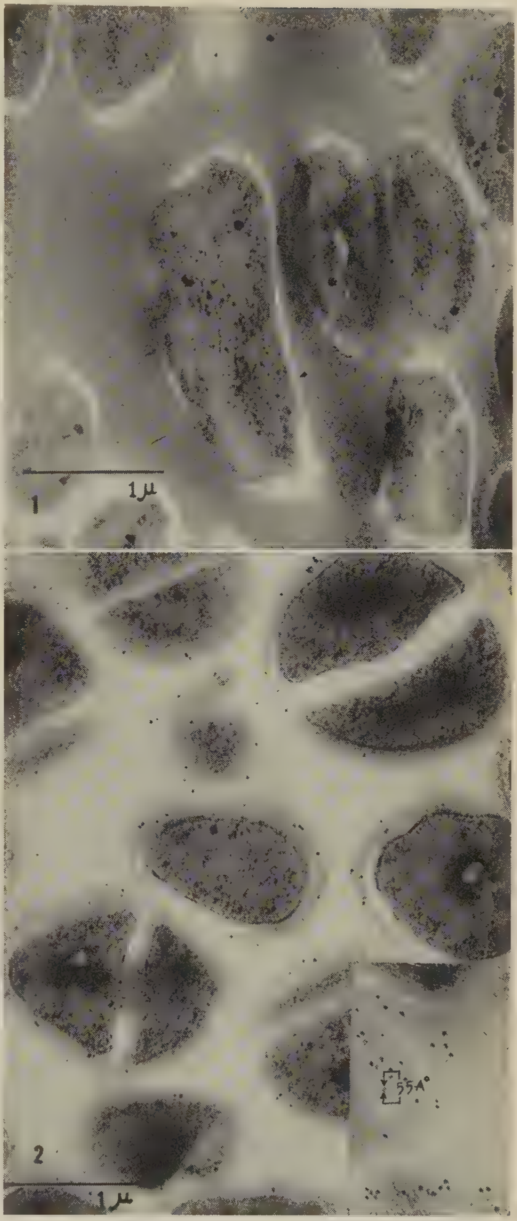

FIG. 1 and 2. Sections of *Staphylococcus aureus* reacted with (1) control conjugate; (2) immune conjugate; (insert) higher magnification showing ferritin molecule.

Farrant(4), in the capsule. In the sections of the organisms in mitosis an occasional spindle-like structure can be observed, as well as what appears to be a double membrane at points of division.

Discussion. Use of electron-dense antibody conjugates presents a new method of observing immune reactions at subcellular level in formalin-fixed preparations. Heretofore formalin fixation has been used infrequently in electron microscopy, as it was believed to reduce ability to obtain good resolution of cellular material. However, the electron-dense material, in what is considered as nuclear region, is well demonstrated in our sections. It has the further advantage of rapidly inactivating any infectious agents used. Although formalin fixation may not be as satisfactory for other tissues as it apparently is for staphylococci, it appears warranted to further investigate its usage in electron microscopy. As unbound ferritin conjugate was completely removed by simple washing, the problem of nonspecific reactions with the immunofluorescent technic was not encountered. This will permit a more critical evaluation of specificity of immune reactions, a problem which has been most troublesome in formalin-fixed tissues. It should now be possible to localize definitely the sites of antigen production as well as viral aggregates in tissues that have been properly fixed with formalin as well as other material at the subcellular level. The number of ferritin molecules around bacteria could probably be increased by changing ratio of antigen-antibody in the reaction.

Summary. A method is described by which immune systems can be studied at subcellular level with the electron microscope, using a ferritin-antibody conjugate. A conjugated immune serum to *Staphylococcus aureus* was applied to homologous organisms, embedded in methacrylate with uranium and thin sections cut. An antigen-antibody reaction was detected by attachment of the ferritin molecule in the capsule-like material. Use of formalin in primary fixation for electron microscopy as well as nonspecific immunofluorescence is discussed.

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Effect of 5-Hydroxytryptamine on Phosphorylase and Glycogen Levels in Muscle Tissue.* (25828)

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Although the isolated uterus of the estrous rat is frequently employed for bio-assay of certain psychopharmacological agents and hormones, little is known concerning the effects of these substances, particularly the former, on glycogen stores and enzyme phosphorylase in uterine muscle. Epinephrine and oxytocin, however, induce a rapid decrease in both uterine glycogen(1) and phosphorylase activity(2). The effects of serotonin (5-OH-tryptamine), lysergic acid and reserpine on these 2 constituents in uterine smooth muscle were investigated. Since serotonin alone produced changes in smooth muscle, its effect was studied also on skeletal, cardiac, and diaphragm muscle.

Methods. Adult female rats were spayed, 7-9 days later injected with 50 μg of estradiol benzoate (Schering Co.) subcutaneously and 48-56 hours later given an intraperitoneal injection of the test compound. Uteri were removed 30-60 minutes later from the anesthetized rats and were frozen and stored as de-

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* Aided by grant from Muscular Dystrophy Assn. and Sage and Sackett Research Funds of Dept. of Zoology, Cornell University.

TABLE I. Phosphorylase Activity in Uterine Smooth Muscle, Diaphragm and Heart.

Treatment*		Exp.			Control		
		P-lase *a*	P-lase *t*	*a/t* ×100	P-lase *a*	P-lase *t*	*a/t* ×100
			Uterus				
Serotonin	(12)	35 ± 2	96 ± 3	36.4 ± 1.0	74 ± 2	116 ± 2	64.0 ± 1.1
Reserpine	(9)	44 ± 2	136 ± 7	33.6 ± .8	46 ± 3	134 ± 5	34.7 ± 2.1
LSD	(12)	62 ± 3	130 ± 2	47.9 ± 2.9	69 ± 3	128 ± 4	54.3 ± 1.7
			Diaphragm				
Serotonin	(10)	119 ± 5	150 ± 3	79.4 ± 1.8	119 ± 3	164 ± 7	72.9 ± 1.4
" & electr. stim.	(8)	33 ± 6	94 ± 7	35.0 ± 5.5	36 ± 4	94 ± 4	39.2 ± 3.6
Epinephr. & " "	(8)	58 ± 5	96 ± 5	60.7 ± 2.7	44 ± 3	108 ± 5	40.5 ± 1.2
			Heart				
Serotonin	(16)	126 ± 9	194 ± 4	64.6 ± 3.2	126 ± 7	189 ± 6	66.4 ± 2.0

* Details in text. Figures in parentheses = No. of rats.

scribed(2). Diaphragms were obtained from male rats, cardiac muscle from both sexes, and all tissues were quickly frozen upon removal. To produce tetanic contraction of the diaphragms prior to removal, electrodes were placed on posterior surface of half of exposed diaphragm of the anesthetized rat while passing a current from electronic stimulator for 20 seconds (frequency 60/sec, duration 1 msec, 10 volts). Serotonin creatine sulfate (California Corp. for Biochem. Res), reserpine (Ciba Pharm. Co.), lysergic acid diethylamide (LSD, Sandoz Pharm. Co.), and l-epinephrine-HCl (Parke Davis Co.), were diluted in water and given in 1 ml volume, intraperitoneally, in doses/100 g body weight. Details of methods of analysis are given; for glycogen(1,3), for uterine (smooth muscle) phosphorylase(2) and for striated muscle phosphorylase(4). Active phosphorylase *a* and total phosphorylase *t* (with added adenylic acid) are reported as μg P liberated from glucose-1-PO$_4$ in 10 minutes at 37°C by 40 mg of uterine tissue, 2.5 mg of diaphragm or 10 mg of cardiac tissue. Results are presented as means ± standard error.

Results. Doses of serotonin sufficient to alter either glycogen or phosphorylase levels were considerably in excess of those used for *in vitro* pharmacological testing. Groups of 4 female rats, pretreated as described, were injected with the following doses of serotonin: 0.1, 0.25, 0.5, 1.0 mg and, after 30 minutes, uterine glycogen levels in mg % were respectively, 291 ± 15, 217 ± 16, 207 ± 17 and 153 ± 40 as compared to 307 ± 7 mg % in controls. To determine if a change in phosphorylase activity would accompany this decrease in glycogen, similarly prepared rats were given 0.5 mg of serotonin and, after 30 minutes, the tissues assayed. Table I shows that a marked decrease in active phosphorylase a and activity ratios occurred. When reserpine and LSD (0.5 mg dose each) were injected into the prepared female rats, within 1 hour, uterine glycogen level was unaltered and phosphorylase activity was unchanged as noted (Table I). The relatively low activity ratios in controls of experiments with reserpine indicate again the variability encountered between successive experiments(2). Phosphorylase a levels in these controls are about twice that found if estrogen had not been given and sufficiently high to permit a lowering of enzyme activity under the proper conditions.

Examination of leg muscles in several rats for changes in either glycogen or phosphorylase revealed that these 3 substances were without effect. A report that serotonin increased uptake of P^{32} in the rat diaphragm (5) suggested a study of this muscle. Serotonin (0.5 mg dose) lowered glycogen levels of diaphragms in male rats from 314 ± 18 to 246 ± 19 mg % in 30 minutes (12 rats, previously starved for a day). When 1 mg doses of serotonin were injected into fed male rats and diaphragms assayed 1 hour later, glycogen decreased from control level of 626 ± 28 to 391 ± 16 mg % (12 rats). There was no change in active phosphorylase and only a slight rise in activity ratios in diaphragms of these rats (Table I).

Except for negative results on phosphorylase levels in the diaphragm, the effects of serotonin were suggestive of those of epinephrine. A more sensitive method for demonstrating epinephrine effects on skeletal muscle phosphorylase requires that muscles of experimental and control animals be electrically stimulated prior to removal(6). To further ascertain that serotonin had no effect on phosphorylase in the diaphragm, the muscle was subjected to this more sensitive assay procedure. Preliminary experiments were made in which doses of 20 μg of epinephrine were given to rats 30 minutes before placing the electrode on the center of the right half of the diaphragm and stimulating for 20 seconds. While the right half was in tetanic contraction, the left half almost always continued its normal respiratory movements (only the right portion assayed). Control rats were treated similarly but without epinephrine. Results in Table I show that epinephrine inhibited the expected decrease in active phosphorylase and maintained activity ratios 50% higher than in controls. When rats were injected with 1 mg doses of serotonin and stimulated as above, phosphorylase activity was not maintained at a level higher than that in controls.

In studying heart muscle, 1 mg doses of serotonin were given to 8 fed rats (4 of each sex) and after 1 hour, the rats were autopsied. An equal number of controls of both sexes were used in the determinations. No sex difference was noted and the results were pooled. Table I shows that serotonin did not alter phosphorylase activity but, surprisingly, heart glycogen increased from a control level of 465 ± 27 to 728 ± 47 mg %.

Discussion. Serotonin, but not reserpine or LSD, lowered uterine glycogen and phosphorylase, both of which are located essentially in the myometrium(7). Although LSD *in vitro* antagonizes serotonin induced uterine contractions(8) and produces overt behavior in the rat, neither smooth nor skeletal muscles were affected. LSD affects carbohydrate metabolism in other ways and in other target organs(9) but there is little information on *in vivo* effects in muscle. The same may be said for serotonin(10) although it produces hyperglycemia without affecting skeletal muscle glycogen(11). High levels of serotonin in the uterus have been produced *in vivo* by administering the precursor, 5-hydroxytryptophan, without inducing abortion or altering uterine activity(12) but it is likely that much higher levels of serotonin are required to affect the glycogen.

Induced changes in uterine smooth muscle glycogen and phosphorylase have so far always been positively correlated(1,2). Acute changes in glycogen in other tissue, (liver, skeletal muscle, heart) have been correlated with changes in phosphorylase but not necessarily in the same direction(6,13,14,15). However, the effect of serotonin was unique, it lowered diaphragm glycogen, elevated cardiac glycogen and in both instances failed to alter phosphorylase activity. Phosphorylase activity may not be rate limiting in all instances of acute changes in tissue glycogen(13). The increase in heart glycogen following serotonin may be the result of increased mobilization and utilization of fatty acids. Preferential utilization of fatty acids over glucose would permit rapid accumulation of glycogen without necessarily requiring a concurrent increase in phosphorylase activity (summary ref. 16). Epinephrine increases cardiac glycogen and phosphorylase activity(14) thus differing from serotonin. Serotonin is also reported to increase phosphorylase and glycogenolysis in the sheep liver fluke(17).

Summary. Serotonin, but not reserpine or lysergic acid (LSD), when given intraperitoneally to rats, decreased uterine (smooth muscle) glycogen levels and phosphorylase activity. Serotonin rapidly decreased glycogen concentration in the diaphragm, rapidly elevated glycogen in heart muscle and in neither instance altered phosphorylase activity. Thus it is possible to produce acute changes in glycogen stores in some tissues without evoking alterations in phosphorylase activity.

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Role of the Pulsatile Thrust of the Aorta in Genesis of Gastric Ulcer.* (25829)

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A jet stream of gastric juice obtained by overnight aspiration from the stomach of patients with duodenal ulcer, projected intermittently over a period of hours, at an ejection pressure of 30 cm of H_2O will produce ulceration in the duodenum of rabbits quite regularly(1). Even perforation of the duodenum has been observed as early as 1.5 hours following commencement of the jet perfusion. On the contrary, a constant flow at the same pressure produces little injury to the duodenum. During the past year, bleeding was observed from the stomach of a patient in whom that viscus had been elevated into the thorax 15 years previously as replacement of the esophagus, for a high-lying cancer. At the recent operation, the pulsatile thrust of the aorta upon the lesser curvature of the stomach, site of origin of the gastric bleeding, was very obvious. This observation suggests the need of assessing the role of the pulsatile aortic thrust in genesis of gastric ulcer.

Methods and materials. Adult mongrel dogs were operated upon under pentobarbital anesthesia. In Group I, through a left subcostal incision, the postero-lateral aspect of the diaphragm was incised exposing the aorta which was dissected free for 3 cm cephalad to the celiac artery. The anterior wall of the stomach was placed behind this segment of the aorta and either sutured back onto itself or to the left lateral abdominal wall (Fig. 1A). In another group of dogs (1A), 30 mg of histamine in beeswax was given intramuscularly once daily to determine whether augmentation of gastric secretion would accelerate ulcer production under these circumstances. In Group II, the stomach was placed behind the aorta of dogs in the same location, with this difference: the aorta in the chest was divided and aortic continuity reestablished by a Teflon graft (Fig. 1B). In consequence, there was no forward blood flow through the segment of the aorta, behind which the fundus of the stomach was placed. To insure absence of pulsations in the portion of the aorta overriding the stomach, an aortic ligature was placed just proximal to the celiac artery. There were 12 dogs each in Group I and Group II, and 6 dogs in Group IA. Upon recovery from operation, all the dogs were fed *ad libitum*.

Results. In Group I, 9 of 12 dogs (75%) died as a result of development of ulceration at the area subjected to the thrust of aortic pulsations. Of these 9 dogs, 5 succumbed

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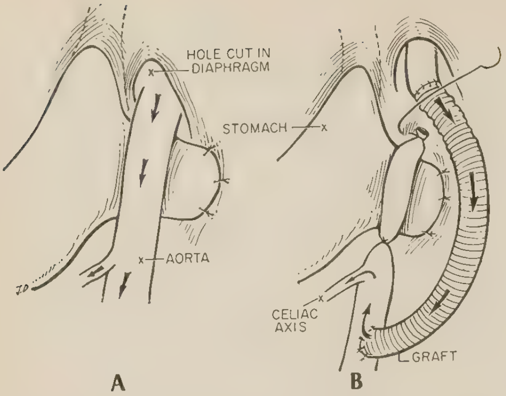

FIG. 1. A. Area of retroaortic placement of stomach. B. Control procedure with segment of aorta overriding stomach without pulsatile flow.

suddenly following a massive upper gastrointestinal hemorrhage secondary to acid-peptic erosion of the gastric and aortic wall with formation of a gastroaortic fistula. Time of appearance of fatal ulceration was between 4 and 17 days with a mean of 10 days. One dog died on the 12th postoperative day without developing an ulcer. The remaining animals are living and well, more than a month following surgery.

In Group IA, 3 dogs succumbed less than 5 days postoperatively, without evidence of gastric ulceration. One of the dogs died 5 days postoperatively, as the result of massive upper gastrointestinal hemorrhage secondary to perforation of a gastric ulcer into the aorta. Another dog died 12 days postoperatively and had a small ulcer in the gastric mucosa overlying the aorta. The remaining animal is alive, 28 days after his operation.

In Group II, there have been 3 deaths, all more than one week postoperatively and no evidence of gastric ulceration has been observed. The longest survivor amongst the 3 fatalities died 22 days following the procedure. The remaining 9 survivors appear to be free from ulcer up to more than 2 months after operation.

Discussion. It is evident from these experiments that the pulsatile thrust of the aorta upon the stomach evokes ulceration of the mucosa with eventual perforation of the viscus, either into the free peritoneal cavity or directly into the aorta with establishment of a gastro-aortic fistula. Local venous obstruction, which has been shown to potentiate development of ulcers(2), is present to the same extent in all groups of animals and thus is not a determining factor. Histamine hypersecretion has not decreased significantly time of appearance of the gastric ulcers.

It is well known that gastric ulcer in man is accompanied frequently by a gastric juice of relatively low volume and digestive power, as compared with the larger volumes, greater acidity and peptic power of gastric juice in patients with duodenal ulcer. Moreover, gastric mucosa is extraordinarily refractory to injury by the gastric juice compared with the very susceptible esophageal epithelium and with duodenal mucosa. In fact, since the time of John Hunter, investigators have continually been asking themselves: why does the stomach not digest itself?

In any position, the stomach of the dog falls away from the aortic pulsations. In man, whether he sits, stands or lies supine, the thrust of aortic pulsations makes itself felt upon his stomach. Perhaps one of the best treatments for gastric ulcer would be to have the patient assume the prone position for long periods of time whenever possible.

Conclusions and summary. 1. Retroaortic placement of the fundus of the dog's stomach is followed by development of gastric ulcer at site of impingement of the aortic thrust upon the gastric wall. Perforation with formation of a gastroaortic fistula was a frequent sequel. 2. It is not unlikely in man that the mid lesser curvature gastric ulcer owes its origin in part to the thrust of aortic pulsations.

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Protection, by Coronary Ligature, Against Isoproterenol-Induced Myocardial Necroses.* (25830)

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Multiple focal myocardial necroses can be produced in various experimental animals by catecholamines, *e.g.*: adrenaline(1,2,3,4) or noradrenalin(5); they are particularly constant and severe after treatment with isoproterenol(6). As we shall see, in rats in which a large infarct is produced by ligature of the left coronary artery, the remaining myocardium becomes unusually resistant to production of focal necroses by subsequent treatment with isoproterenol.

Methods. One hundred female Sprague-Dawley rats, with mean initial body weight of 230 g (range: 180-339 g), were subdivided into 5 equal groups and treated as indicated in Table I. *Ligature of left coronary artery* was performed near the origin of the vessel—without pressure respiration, according to a simplified technic described elsewhere(7)—on the first day of experiment. *Sham coronary ligature* was carried out by the same procedure, except that the ligature was placed in cardiac muscle adjacent to the left coronary artery. Since the sham coronary ligatures do not produce myocardial infarction, they are not as stressful as is occlusion of the coronary vessel. Consequently, additional groups of controls were submitted to particularly severe stress to determine the possible effect of intense systemic damage as such. These rats were subjected either to *forced restraint* by tying them to a board for 24 hours on the first day and 7 hours on the fifth day according to a previously described technic(5), or to denervation of their 4 extremities by *transection of the motor nerves* on the first day of experiment, by using a procedure also described elsewhere(5). *Isoproterenol* (Winthrop Laboratories, N. Y.) was administered at the dose of 100 mg/100 g body weight, in 0.5 ml of water, subcutaneously, twice daily, on sixth and seventh days of experiment. The diffuse lesions caused by isoproterenol cannot always be seen clearly on fresh specimens; hence, the *severity of the changes* was appraised on histologic sections stained with the PAS procedure. Intensity of the focal necroses in right ventricle and septum was graded in terms of an arbitrary scale of: 0 (no lesion), 1 (slightest detectable lesion), 2 (moderate lesion), and 3 (most severe lesion). The means of these values are given in Table I with their standard errors.

Results. The lesions produced by isoproterenol in the rats that received no other treatment (Group 1) were constant and severe; they consisted of multiple foci of muscle necrosis secondarily infiltrated by inflammatory cells, particularly macrophages and fibroblasts. The lesions varied in size and location, but showed a definite predisposition for subendocardial layers (Fig. 1). There was also hyaline necrosis. In the walls of many larger arteries, there appeared homogeneous PAS-positive deposits (Fig. 2) and edema developed within the myocardium, especially around the affected arteries. Lesions of essentially the same character and intensity were seen in the rats with sham coronary ligatures (Group 3) and in those previously exposed to the stress of forced restraint

TABLE I. Protection, by Coronary Ligature, against Isoproterenol-Induced Myocardial Necroses, in 5 Groups.

Treatment*	Disseminated cardiac necrosis (scale 0-3)	Mortality (%)
None	1.7 ± .20	30
Coronary ligature	.2 ± .10	10
Sham coronary ligature	2.0 ± .19	25
Restraint	2.0 ± .17	0
Motor denervation	2.3 ± .17	20

* In addition to treatments listed in this column, rats of all groups received *isoproterenol* as indicated in text.

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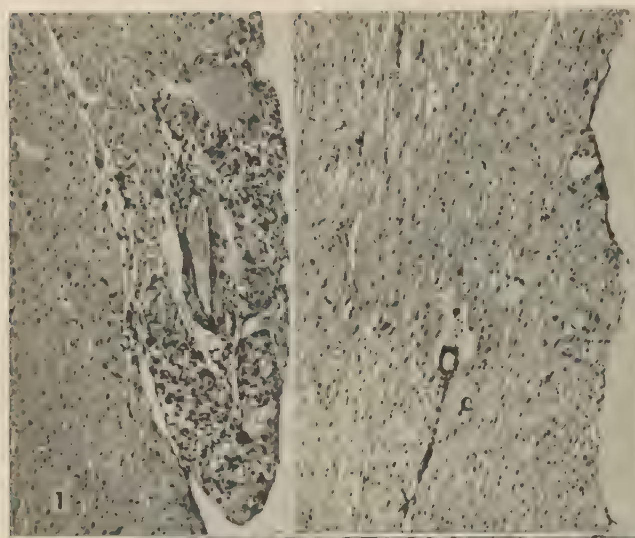

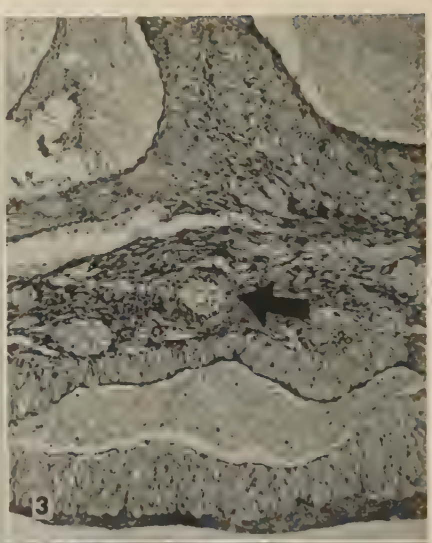

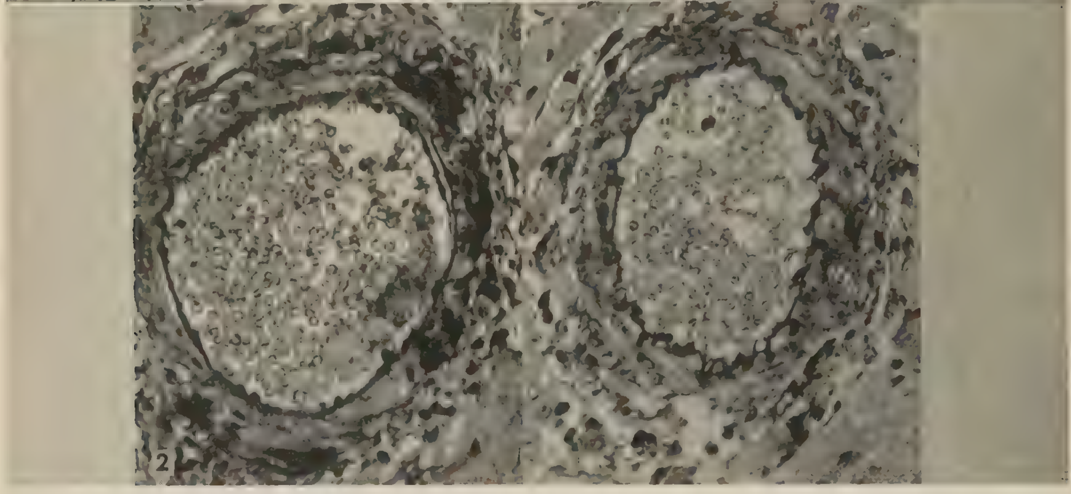

FIG. 1. Subendocardial muscle strata, following treatment with isoproterenol. *Left*, necrosis with inflammatory infiltration, in otherwise untreated rat. *Right*, no necrosis in corresponding region of myocardium, in rat with coronary ligature (PAS × 73).

FIG. 2. Medium sized coronary artery, following treatment with isoproterenol. *Left*, strongly PAS-positive (dark) homogeneous deposits, in an otherwise untreated rat. *Right*, essentially normal arterial structure, in a rat with coronary ligature (PAS × 266).

FIG. 3. Section through the entire thickness of left ventricular wall, in a rat treated with isoproterenol after ligature of left coronary artery. Almost the entire myocardium has been replaced by scar tissue, except for a thin subendocardial layer (top) and a narrow cuff around the large vein (bottom). Around the artery (arrow), no normal myocardium persists (PAS × 73).

(Group 4) or motor denervation (Group 5).

In rats in which the left coronary artery had been occluded 6 days before isoproterenol treatment, virtually the entire wall of the left ventricle underwent coagulation necrosis. Only a narrow layer of surviving myocardium (about 3-5 myofibrils in thickness) remained as an uninterrupted lining along the subendocardial surface of the infarcted area. A similar but less regular lining was also noted just underneath the epicardium that covered the necrotic patch, as well as around the veins (but not around the arteries) within the infarct itself. As indicated elsewhere(7), these muscle strata probably survive because they derive nutrition from the free blood within the ventricle, pericardial vessels, and veins that merely traverse the infarct (Fig. 3). Disseminated necrosis or arterial hyalinization, characteristic of isoproterenol overdosage, was not observed anywhere in the surviving myocardium, except in 2 of the 20 animals in Group 2, and even here, the lesion was barely detectable. Mortality due to isoproterenol intoxication occurred only on 7th day; it was not markedly altered by coronary ligature, perhaps because the detrimental effect of the large infarct nullifies protection against focal necrosis.

Discussion. Evidently, ligature of the left coronary artery near its origin offers considerable protection against subsequent induction of diffuse myocardial lesions by isoproterenol. This protection dose not appear to be due to handling the heart at time of operation, since sham coronary ligatures are ineffective in this respect. Systemic stress may also be excluded as the protective agent. Rats recover from such coronary ligatures almost completely within one or 2 days, as judged by their appetite and general clinical condition; besides, neither forced restraint nor motor denervation —both of which elicited much more pronounced manifestations of stress (adrenal enlargement, thymic involution, loss of body weight)—offers protection against isoproterenol-induced disseminated cardiac necroses. After coronary ligature, systemic blood pressure remained essentially within normal limits; hence, a pre-existent hypotension likewise could not be held responsible for the isoproterenol resistance of surviving myocardium.

The mechanism of this protection remains obscure. Perhaps, when in the state of compensatory hypertrophy (such as occurs after infarction), cardiac muscle becomes insensitive to toxic action of isoproterenol. The resistance may also be due to hemodynamic changes within the myocardium and its vessels, or to absorption of chemical compounds from the infarcted muscle. It remains to be seen, furthermore, whether this protection is specific for isoproterenol and related catecholamines. Additional work will be necessary to clarify these points, but the protective effect of coronary ligature appears to be well established.

Summary. Experiments on rats indicate that, following occlusion of the left coronary artery near its origin, the myocardium outside resulting infarct becomes unusually resistant to production of diffuse cardiac necroses by isoproterenol.

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Biological Activity of Some 6α-Fluoro and 16α-Methyl C-21 Steroids.* (25831)

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Discovery that cortisone was effective as treatment for inflammatory conditions(1) has led to attempts to increase efficacy by chemical modifications. Notable among these have been Δ^1, 2α-methyl, 6α-methyl, 9α-halo, 16α-hydroxy and, recently, 16α-methyl(2,3,4,5,6,7,8,9,10,11). These changes have also been used in combination in attempts to alter some of the undesirable biological effects resulting from some of the single modifications. The present communication describes the influence of the 6α-fluoro and 16α-methyl groups, alone

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* Steroids kindly supplied by Chemistry Dept., Upjohn Co.

TABLE I. Anti-Inflammatory, Liver Glycogenic and Sodium Retaining Activities of Some 6-Fluoro and 16-Methyl C-21 Steroids.

	Activity		
Parent compound	Anti-inflammatory × hydrocortisone	Liver glycogen × hydrocortisone	Sodium retention × DCA
Hydrocortisone	1.0	1.0	<.02
16α-methylhydrocortisone	.8	1.4	”
Prednisolone	3.1(6)	3.0(6)	”
16α-methylprednisolone acetate	8.7	24.0	”
9α-fluorohydrocortisone acetate	8.0	12.6	5.0
9α-fluoro-16α-methylprednisolone	164.0	251.0	<.02
2α-methylhydrocortisone acetate	2.8	5.8(3)	2.7
9α-fluoroprednisolone acetate	16.5	50.0	20.
6-Fluoro compound			
6α-fluorohydrocortisone acetate	8.7	10.9	Slight retention
6α-fluoro-16α-methylhydrocortisone	6.1	36.0	<.02
6α-fluoroprednisolone acetate	25.0	100.0	Slight retention
6α-fluoro-16α-methylprednisolone acetate	50.0	150.0	<.02
6α,9α-difluorohydrocortisone acetate	8.0	63.0	4.0
6α,9α-difluoro-16α-methylprednisolone acetate	424.0	677.0	<.02
6α-fluoro-2α-methylhydrocortisone acetate	23.0	60.0	3.0
6α,9α-difluoroprednisolone acetate	66.0	443.0	2.5

and in combination with other potentiating groups on the anti-inflammatory, liver glycogenic, and mineralocorticoid activities of hydrocortisone.

Materials and methods. Anti-inflammatory test. Granuloma pouch method(12) was used to determine anti-inflammatory potency of the various steroids. In all cases, unknowns were tested in parallel with hydrocortisone on a 3-dose basis with a minimum of 14 animals per dosage group. Potency calculations were carried out by a standard U.S.P. method(13). Steroids were injected subcutaneously in CMC vehicle.† Animals were intact Sprague-Dawley females, weighing 150-160 g. *Liver glycogen deposition test.* Liver glycogens were determined by the anthrone method(14) on tissues removed 7 hr following subcutaneous injection in CMC vehicle. Three doses of unknowns were assayed in parallel with 3 doses of standard with at least 10 rats per group. Potency ratios were calculated by the method of Irwin(15). *Sodium retention test.* This test(16) measures the influence of compounds on sodium and water excretion in salt and water loaded, adrenalectomized rats over a 4 hr period, following a single subcutaneous injection of the compound. Compound is administered as an oil solution or suspension, depending upon concentration and solubility. Compounds possessing little or no sodium retaining activity were tested at a dose of 500 μg and compared to a dose response curve obtained with desoxycorticosterone. When compounds possessed a significant degree of sodium retaining activity, they were tested in parallel on a multiple dose basis with the standard. Potencies in the cases of active compounds were calculated by the Irwin method(15).

Results. Anti-inflammatory potency of the steroids included in this study was increased by 6α-fluoro in all cases except 9α-fluorohydrocortisone (Table I, Fig. 1). 6α-Fluoro increased the glycogenic potency of all compounds in this series. Variable results were obtained on sodium excretion in presence of 6-fluoro since, in some cases there was an increase, in others no change, while sodium retention of one compound (9α-fluoro-prednisolone) was markedly depressed when 6α-fluoro was present (Table I). Influence of the 6α-fluoro on sodium retention is different than that obtained with 6α-methyl which consistently decreased the activity of sodium retainers or in the case of 2α-methyl or 9α-fluoro which generally increased sodium retention(6). Reason for the variable results of 6α-fluoro on anti-inflammatory and sodium retaining activity is difficult to interpret but

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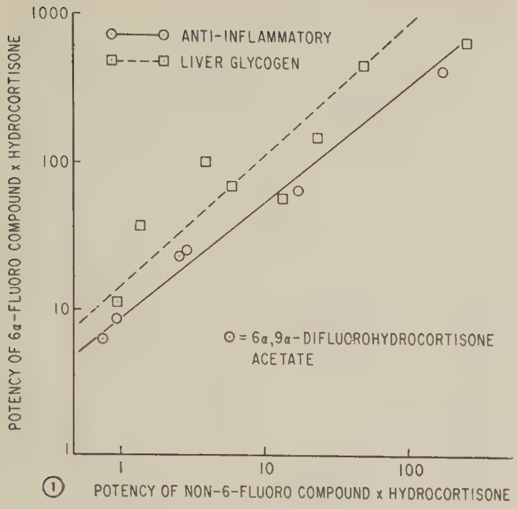
† 0.5% carboxymethylcellulose, 0.5% Tween 80, 1.5% benzyl alcohol, 0.9% sodium chloride.



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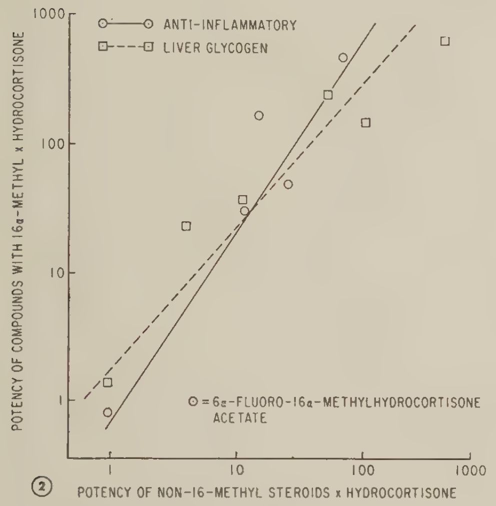

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FIG. 1. Graphic representation of data in Table I showing relationship of 6-fluoro and non-6-fluoro steroids on anti-inflammatory and liver glycogenic potencies.

FIG. 2. Summary of relationship of 16-methyl and non-16-methyl compounds on anti-inflammatory and liver glycogenic activities.

may be due to 1) differences in physical properties of compounds which produce different results due to characteristics of a given test, or 2) actual changes in inherent activity which is manifested by different effects at the target organs.

16α-methyl is also not a universal potentiator of glycogenic or anti-inflammatory activity (Table I, Fig. 2). This is in contrast to other published reports(5,7). When the parent compound was a sodium retainer, the compound with the 16α-methyl generally possesses less sodium retaining activity. In one case where the parent compound was inactive, the presence of 16α-methyl did not cause any change in sodium retaining activity. It is therefore concluded from the series studied here that 16α-methyl does not cause sodium retention but actually depresses sodium retaining activity of sodium retainers.

Results reported here show that it is not yet possible to predict accurately the effects of chemical modifications on biological activities of steroids. Chemical changes in certain combinations can be expected to produce variable alterations in quantitative activities. It has been our experience in certain instances, however, when a single change increases the anti-inflammatory or glycogenic potency of a compound, it does not decrease potency when in combination with other potentiators.

One compound (6,9-difluoro-16α-methylprednisolone) is one of the most potent anti-inflammatory or glycogenic steroids tested. When this is considered along with the fact that the compound does not possess significant sodium retention at doses which are anti-inflammatory, it shows that one biological activity can be altered independently of another. This compound is therefore potentially useful clinically in inflammatory conditions.

Observations of marked increase in anti-inflammatory activity with no change or decrease in sodium retaining activity lead to optimism in testing new steroids for differential activities. Since it is possible to reduce sodium retention activity while retaining glycogenic or anti-inflammatory potency, it may also be possible to dissociate, by chemical modification, protein catabolic and ulcerogenic activities from anti-inflammatory effects.

Summary. The anti-inflammatory, liver glycogenic, and sodium retaining activities of a series of 6α-fluoro and 16α-methyl steroids and their parent compounds were determined. The 6α-fluoro substitution increased liver glycogenic activity in all compounds studied, and anti-inflammatory potency in all but one

of compounds. This modification also produced variable effects on sodium retention. The 16α-methyl group did not consistently increase liver glycogenic or anti-inflammatory activities. Sodium retention effect of the corticoids was not increased by 16-methylation if parent compound did not possess mineralocorticoid activity. In examples where parent steroid was a sodium retainer, addition of 16α-methyl group caused reduction of this activity.

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Zn^{65} Uptake by Rat Dorsolateral Prostate as Indicator of I.C.S.H. Activity.*

(25832)

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Previous investigations have shown that Zn^{65} uptake by dorsolateral prostate (DLP) of the mature rat reflects high natural zinc content of the gland(1). In contrast the ventral lobes of prostate, which are low in natural zinc content(2), do not take up Zn^{65} appreciably(1). It was also demonstrated that amount of Zn^{65} concentrated by DLP depended upon androgen level. Zn^{65} uptake was low in DLP of immature rats and did not reach maximum levels until animals were 14-16 weeks of age (3). This also paralleled other reports on increase in natural zinc content of the rat DLP with age(4). Further experimentation showed that Zn^{65} uptake in DLP of the mature rat was lowered by castration, but could be maintained at control levels by administration of suitable doses of testosterone propionate(3,5). Even more striking fall in Zn^{65} uptake was noted following hypophysectomy, but Zn^{65} uptake levels could be maintained by administration of appropriate doses of either chorionic gonadotrophin or testosterone propionate(5,6). One biological indicator of I.C.S.H. activity frequently used is based on enlargement of the ventral prostate in hypophysectomized rats(7). In view of availability of purified I.C.S.H.† experiments

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* Supported by grant from U. S. Atomic Energy Comm.

† Generous supplies of I.C.S.H. (L.H.) were furnished by Endocrinology Study Section, Nat. Inst. Health.

were set up to determine (a) to what extent this hormone would enhance the capacity of DLP to concentrate Zn^{65} above hypophysectomized levels, and (b) which of the following measurements would be the most sensitive indicator of I.C.S.H. activity in hypophysectomized rats, *i.e.*, ventral prostate weight, DLP weight, Zn^{65} uptake/mg DLP, or total Zn^{65} uptake by DLP.

Methods. Hypophysectomized male Wistar rats (16 weeks of age, weighing 300 to 350 g) were purchased from Charles River Breeding Labs, Brookline, Mass. On 5th day post-surgery I.C.S.H. was administered to 20 hypophysectomized rats at 0.2 mg/rat. Injections were made intramuscularly in 0.2 ml physiological saline. Daily injections were continued for 6 days. Ten hypophysectomized rats administered 0.2 ml physiological saline daily served as controls. On 6th day of injections, Zn^{65} was administered intracardially at 0.04 μc/g as outlined previously(8). Twenty-four hours later the animals were sacrificed and ventral prostates removed. At the same time DLPs were dissected by technics described previously(9). After drying overnight at 110°C ventral prostates and DLPs were weighed. Zn^{65} uptake in DLP was determined in well-type scintillation counter as outlined earlier(8). The following measurements were made: (a) ventral prostate weight, expressed as mg dry weight; (b) DLP weight, expressed as mg dry weight; (c) Zn^{65} uptake/mg DLP, expressed as counts/minute/mg tissue; and (d) total Zn^{65} uptake in DLP, expressed as total counts/minute. All rats used were inspected grossly for completeness of hypophysectomy. None showed any pituitary remnants. The animals were housed under constant conditions of temperature, humidity and lighting (12-hour light, 12-hour darkness cycle). Experiments were conducted during March and April, 1960(5,8).

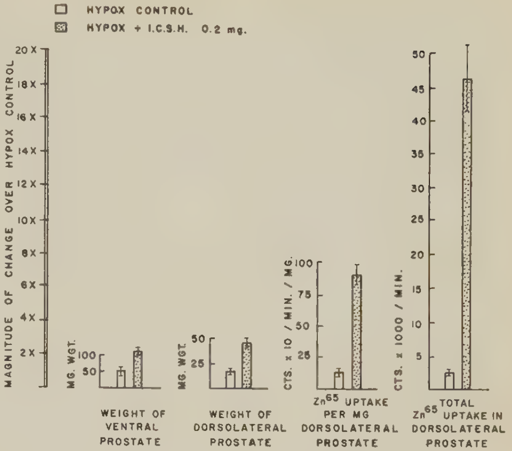

FIG. 1. Effect of daily administration of 0.2 mg of I.C.S.H. to hypophysectomized rats on (a) ventral prostate wt, (b) DLP wt, (c) Zn^{65} uptake/mg DLP, and (d) total Zn^{65} uptake in DLP. S.E. of mean are shown.

Results. Fig. 1 shows that daily administration of 0.2 mg of I.C.S.H. (a) increases the weight of ventral prostate 2.3-fold over the hypophysectomized control ($P<0.001$), (b) increases the weight of DLP 2.7-fold over the hypophysectomized control ($P<0.0001$), (c) increases Zn^{65} uptake/mg DLP 6.7-fold over the hypophysectomized control ($P<0.0001$), and (d) increases total Zn^{65} uptake by DLP 18.4-fold over the hypophysectomized control ($P<0.0001$).

Summary. Our purpose was to determine which of the following measurements would be the most sensitive indicator of I.C.S.H. activity in hypophysectomized rats, *i.e.*, weight studies of ventral prostate and of DLP, or functional studies of capacity of DLP to concentrate administered Zn^{65}. 0.2 mg of I.C.S.H. daily increased ventral prostate weight and DLP weight 2.3-fold and 2.7-fold, respectively, over hypophysectomized control values. Measurement of functional capacity of DLP to concentrate Zn^{65} was a much more sensitive indicator of I.C.S.H. activity as indicated by the following: 0.2 mg I.C.S.H. increased Zn^{65} uptake/mg DLP 6.7-fold over hypophysectomized control value, and increased total Zn^{65} taken up by the DLP 18.4-fold over hypophysectomized control value.

The authors express appreciation to Dr. R. T. Hill for his interest.

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In vitro and *in vivo* Radioautography in Recognition and Localization of Gastric Cancer.* (25833)

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Malignant neoplasms have been shown to take up a greater amount of radioactive phosphorus than does normal tissue(1,2). Initial studies with *in vitro* radioautography provided the basis for extending the use of this property of radioactive phosphorus to clinical detection of occult gastric malignancy by a method of *in vivo* radioautography. *In vitro* radioautographs were obtained of 25 resected gastrointestinal specimens following intravenous administration of 500 μc P32, 12 to 24 hours prior to surgery in patients with suspected carcinoma of the gastrointestinal tract. The excised organ, directly following removal, was opened in its long axis and the mucosal surface was washed with saline solution. In a dark room, the excised tissue was then placed on an 8 by 10″ x-ray film with the mucosal surface down and separated from the film by a piece of Saranwrap to prevent moisture from the specimen affecting the photographic emulsion. Care was necessary to smooth out all mucosal folds so that only a single thickness of the mucosa was exposed to the x-ray film. The film and specimen were then placed in a black lined box and retained in the refrigerator for 12 to 16 hours, following which the x-ray film was developed. Malignant neoplasms consistently produced a black area signifying increased radioactivity (Fig. 2). Confirmation of the black areas as owing to the malignant lesion was obtained by super-imposing each specimen on the developed film. Benign ulcers demonstrated a lighter area than normal mucosa, thus suggesting a lower P32 uptake of that lesion than in adjacent healthy tissue (Fig. 1). Correct positives were noted in all 18 gastric, colonic, or rectal cancers and correct negatives in the remaining 7 cases. There were no false positive or false negative results except for area of early polypoid hyperplasia adjacent to gastric carcinoma which presented a false positive (see arrow, Fig. 2).

Methods and materials of in vivo *radioautography.* Patients with suspected gastric malignancy or with precursor conditions, such as pernicious anemia or achlorhydria, were given 500 μc of radioactive phosphorus in the form of sodium phosphate orally, 12 hours prior to study. A thin latex balloon (condom) coated on the inside surface with a highly sensitive photographic emulsion† on an elastic latex-base and attached to a No. 14 French nasogastric tube was then passed into the stomachs of these patients, inflated with air to about 600 ml, left in place for 6 hours, then removed and developed. A blackened area on the balloon denoted proximity of the photo-sensitive coated balloon to an area which had an increased uptake of radioactive phosphorus, indicating probable presence of a malignant tumor (Fig. 3).

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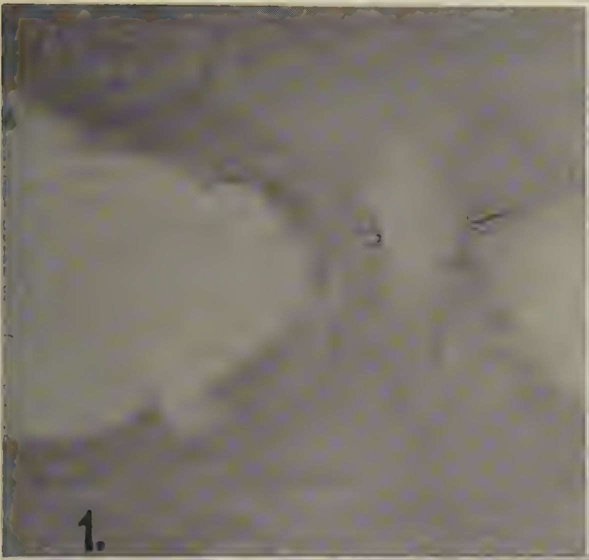
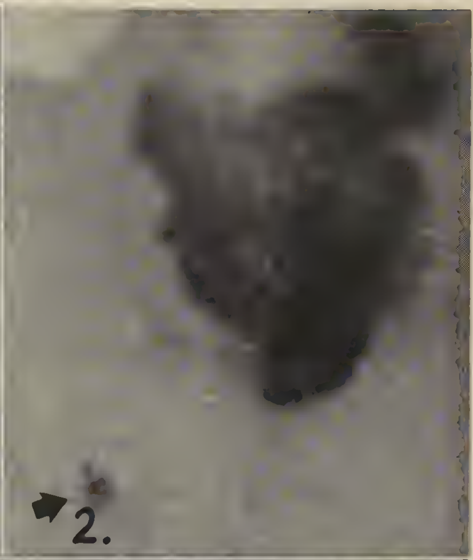
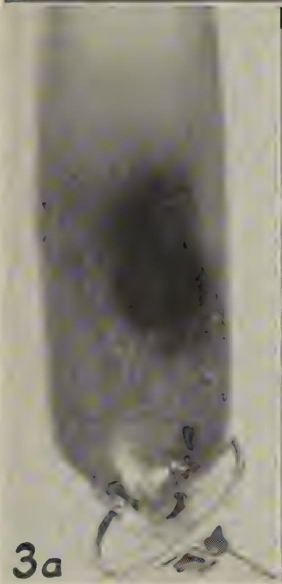
* Supported by the Donald J. Cowling Research Fund, Malignant Disease Research Fund and the American Cancer Society.

† Eastman Kodak Co., Rochester, N. Y.







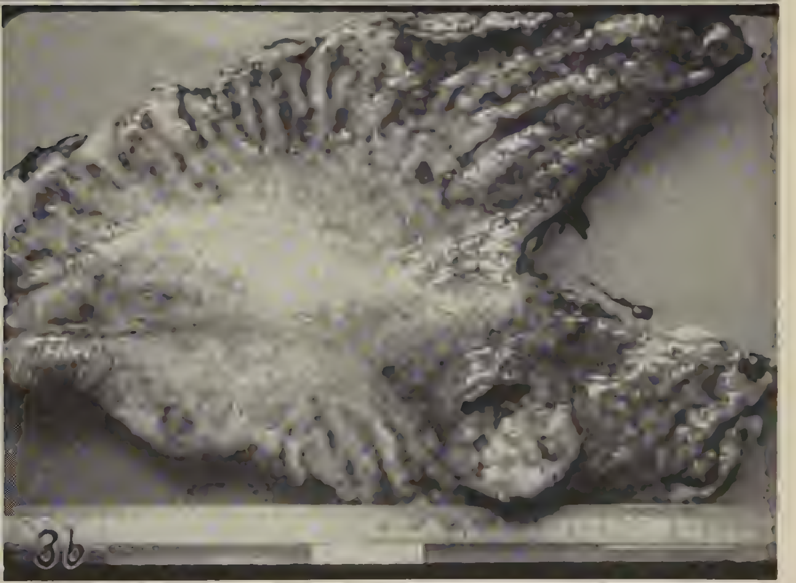


FIG. 1. *In vitro* radioautograph of gastric ulcer with punched out crater—usual finding in benign gastric ulcer.

FIG. 2. *In vitro* radioautograph of a large gastric cancer (dark area). Arrow points to area of polypoid hyperplasia.

FIG. 3a. *In vivo* radioautograph of a patient with gastric cancer. X-ray studies were also positive for gastric cancer.

FIG. 3b. Resected stomach showing malignancy.

Results. Two groups of patients have been screened: 1. A symptomatic group of 18 patients; 2. An asymptomatic group of 40 gastric cancer precursors. This latter group includes 11 patients with pernicious anemia, 5 with gastric polyps, 2 patients with hypertrophic gastric rugations, and 22 patients with long-standing achlorhydria on histamine stimulation. In none of the asymptomatic groups of patients was any suggestion of malignancy uncovered by *in vivo* radioautography. It is proposed to rescreen these patients once a year by the same means.

In the symptomatic group, of 7 patients

found at operation to have microscopically verified gastric cancer, all gave evidence on development of the photosensitive balloons of having a gastric malignancy by *in vivo* radioautography. In 6 of the 7 patients a correct preoperative roentgen diagnosis of malignancy was made. In 4 of these 6 patients repeated x-ray studies were necessary to establish the presence of a lesion. In the remaining patient, the roentgen diagnosis was benign ulcer; the malignancy in this patient was correctly identified preoperatively by *in vivo* radioautography.

Two additional symptomatic patients, by roentgenographic examination, were diagnosed as having gastric cancer; however, *in vivo* radioautographs were consistent with benign gastric disease. Both, at operation, were found to have normal stomachs with cancer in adjacent organs.

Of 11 symptomatic patients with benign gastric ulcer studied, 9 were diagnosed by the radiologist as benign; one lesion, erroneously diagnosed as gastric cancer by *in vivo* radioautography, was not observed on roentgen study; in the remaining patient, the radiological impression was that the lesion was probably malignant. This patient had a negative *in vivo* radioautograph. At operation a benign gastric ulcer was found and excised. Of these 11 patients with benign gastric ulcer, *in vivo* radioautography was unequivocally negative in 7 instances, definitely positive in 1, and questionable in 3.

Discussion. Roentgen studies, the best available diagnostic agency in recognition of gastric cancer, have limitations. X-ray studies have been notably disappointing, moreover, in detection of early asymptomatic mucosal gastric cancers. Inasmuch as the success of *in vivo* radioautography is contingent solely upon the greater affinity of cancer for radioactive P32 over that of the contiguous normal gastric tissue, there is good reason to believe that *in vivo* radioautography will come to supersede x-ray studies in detection of silent mucosal gastric cancer.

In punched-out gastric ulcer craters, devoid of gastric mucosa, *in vivo* radioautographic studies have been consecutively negative. The false positives have been observed in healing gastric ulcers. The presence of an actively regenerating tissue may be responsible. This aspect of the inquiry needs further elucidation. Neither in the atrophic gastritis of pernicious anemia nor in patients with hypertrophic gastric rugae were areas of increased absorption of radioactive P32 observed with *in vivo* balloon radioautography.

It is believed that this same technic with minor alterations can be extended to include examination of other organs such as the cervix, rectum, breasts, and prostate, thus permitting multiple screening from one tracer dose of radioactive phosphorus.

Conclusion. 1) A technic of *in vivo* gastric radioautography is described, employing a balloon (condom) coated with a photosensitive emulsion. Its use in recognition of gastric cancer is outlined. 2) The method served to identify correctly and uniformly the presence of gastric malignancy. There have been no false negatives. The only false positives observed have been in healing gastric ulcers; the nature of this discrepancy needs to be resolved by a larger experience and continued study of the problem.

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Non-Occurrence of Spirochetes in Cerebrospinal Fluid of Patients with Multiple Sclerosis.* (25834)

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Ichelson(1,2) alleges to have cultured a spirochete, *Spirochaeta myelophthora* (Steiner), from the spinal fluid of patients with multiple sclerosis. She attributes etiologic significance to this organism(6). Both denials(3,7) and support(4) of her claims have appeared. Martin and his associates(5), in the most extensive studies done to date, have not only not confirmed Ichelson's claims, but have shown the organism in question to be a contaminating *Vibrio tyrogenus* derived from the bottled distilled water which Ichelson has used in manufacture of her media. Since the question of the etiology of multiple sclerosis is important, presentation of further non-confirmatory data is warranted. The purpose of the present note is to add a series of negative findings. The present study was "blind," in that spinal fluid samples were obtained from the Multiple Sclerosis Clinic, Dept. of Neurology, Hospital of the Univ. of Pennsylvania, Philadelphia, through the courtesy of Dr. Melvin Thorner, who sent them designated by code numbers, without name or diagnosis, to the laboratory. Cultural and other studies on all samples were done with no knowledge of the clinical source from which they were derived. Following completion of the series, the results obtained on each numbered fluid were combined with the appropriate name and diagnosis, then supplied by Dr. Thorner.

The *materials and methods* used were identical to those described by Ichelson(1), except that doubly distilled water, made in this laboratory, was used in place of the bottled distilled water recommended by Ichelson. The 2 distillations were done in 2 Stokes stills operating in tandem.

Results of culture studies are shown in Table I.

TABLE I.

	Spinal fluids	Positive cultures	Negative cultures
Controls	32	0	32
Probable multiple sclerosis	3	0	3
Multiple sclerosis	22	0	22
Total	57	0	57

The table shows the completely negative results. They indicate that no spirochete was found associated with the multiple sclerosis cases in this series.

All cultures have been held a minimum of 18 months. All primary cultures were periodically checked by means of phase contrast microscopy, as were all subcultures. Had a delayed growth occurred, it would have been found. Our results support those of Needham and associates(3), and Martin *et al.*(5), and contradict those of Ichelson(1,2) and Myerson *et al.*(4).

Summary. Spinal fluids from 32 patients not having multiple sclerosis, 22 patients having multiple sclerosis, and 3 patients having probable multiple sclerosis, were cultured according to the method of Ichelson except that the distilled water used was made in our laboratories. All cultures, carried a minimum of 18 months, were negative for spirochetes.

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Urinary Excretion of Folic and Folinic Acids in Normal Adults.* (25835)

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Several reports have appeared giving values for urinary excretion of folic (FA) and folinic (CF) acids in human subjects in health and disease(1-7). However, no report has appeared in which a large number of normals has been studied. Consequently, it has not been possible to evaluate statistically resting levels of FA and CF excretion. Furthermore, many values reported for FA excretion are not specific for FA but include also CF(1-3). The purpose of this study is to establish urinary excretion values for both FA and CF in a large number of normal adults.

Materials and methods. A pad-plate technic was used for microbiological assay of FA and CF(8) similar to the riboflavin assay reported in detail by Simpson(9). The assay procedure involves addition of samples to paper-pads which are then placed on an agar media containing the growth factors for the organism (with omission of FA or CF) and seeded with the assay organism. The plates containing the agar are then incubated for 24 hr at 37°C, at which time circular zones of growth are seen around the pads. Diameter of the zone is measured to the nearest 0.1 mm with a vernier caliper, and a standard curve constructed plotting average mm of growth against concentration of FA or CF in millimicrograms per ml. Standard curves were run with each determination. Four determinations were made on each unknown sample, and average value recorded. Average error of the method was found to be 10% for CF assay and 12% for FA assay. The CF assay organism used was *Leuconostoc citrovorum* (A.T.C.C. #8081)‡ while the FA assay organism was *Streptococcus fecalis* (A.T.C.C. #8043). Commercially available folic acid was used as the FA standard, while Calcium Leucovorin‡ was used as the CF standard. The latter was corrected for presence of the inert isomer(10) by multiplying the obtained CF result by 0.5. The CF activity for *L. citrovorum* (corrected) was subtracted from total activity for *S. fecalis* to ascertain FA activity, since *S. fecalis* responds equally well to FA and CF. The subjects studied were 51 healthy adults ranging from 22-63 years, and the great majority were medical students. Twenty-four hour urine samples were collected in brown bottles, pH adjusted to 6.8 and aliquots stored at 4°C prior to assay. No attempt was made to protect labile folates from oxidative-degradation. Determinations were made on some subjects from day to day to assess daily fluctuations. All subjects were on unrestricted dietary regimes.

Results. The 24-hour urinary excretion of FA and CF in these subjects is given in Table I. Excretion of FA ranged from undetectable levels to 5.58 μg per 24 hr with a mean value

TABLE I. Urinary Excretion of FA and CF in 51 Normal Adults.

μg/24 hr		μg/24 hr		μg/24 hr	
CF	FA	CF	FA	CF	FA
.19	2.20	.40	.40	.64	2.04
.22	.21	.40	.66	.66	.16
.24	.81	.41	1.06	.66	.22
.25	.43	.43	.71	.67	3.01
.28	4.37	.45	2.85	.68	1.80
.29	3.78	.47	.15	.72	.08
.30	4.34	.47	1.11	.73	Un
.30	.35	.47	.91	.88	.55
.30	.30	.48	.40	.89	Un
.31	.70	.49	1.00	.92	5.58
.32	.46	.50	.29	.93	Un
.35	.13	.51	Un*	1.00	1.25
.36	.36	.53	.53	1.22	1.00
.36	.36	2.54	.50	1.29	4.72
.37	.12	.57	.72	1.31	.79
.39	.16	.57	Un	1.33	.91
.39	2.23	.62	.02	1.60	5.10

CF: Mean = .59 μg/24 hr ± .47 stand. dev.
Range = .19–1.60 μg/24 hr.

FA: Mean = 1.17 μg/24 hr ± 1.92 stand. dev.
Range = undetectable–5.58 μg/24 hr.

* Un = undetectable.

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‡ Generously supplied by Dr. H. P. Broquist, Lederle Labs., Pearl River, N. Y.

TABLE II. Daily Excretion of FA and CF.

Subject	Day	μg CF/24 hr	μg FA/24 hr
JD	1	.30	4.34
	2	.49	1.00
	3	.64	2.04
KI	1	.30	.30
	2	.35	.13
	3	.41	1.06
SM	1	.37	.12
	2	.40	.66
HA	1	.39	.16
	2	.47	1.11
JH	1	.67	3.01
	2	.92	5.58
ME	1	1.22	1.00
	2	.57	.72
MC	1	1.29	4.72
	2	1.33	.91

of 1.17 and a standard deviation of ± 1.92; while CF excretion ranged from 0.19–1.6 μg per 24 hr with a mean of 0.59 and a standard deviation of ± 0.47. These results are comparable to those in other studies(4-7). The results of 24 hour urinary excretion of FA and CF in 7 subjects studied on consecutive days are given in Table II. Variation in FA and CF excretion from day to day may be a result of a varying dietary intake. In general, FA excretion varied from day to day and from subject to subject much more widely than CF excretion.

It has been reported that the bulk of CF activity in urine is in an oxygen-labile form which is degraded rapidly in air(11). No attempt was made in this investigation to protect these forms from oxidation, and the results reported here are representative only of oxygen-stable FA and CF.

Summary. The 24 hour urinary excretion of folic (FA) and folinic acid (CF) was determined in 51 healthy adults on unrestricted diets, using pad-plate microbiological assay. The 24 hour excretion of FA ranged from undetectable levels to 5.58 μg/24 hours with a mean of 1.17 and a stand. dev. of ± 1.92; while 24 hr excretion of CF ranged from 0.19-1.6 μg/24 hr with mean of 0.59 and a stand. dev. of ± 0.47.

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Isolation of a Virus Closely Related to Powassan Virus from *Dermacentor andersoni* Collected along North Cache la Poudre River, Colo. (25836)

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In Sept. 1958, a 5-year-old boy from Powassan, Ontario, died of an acute encephalitis, and McLean and Donohue(1) isolated a virus from his brain. The agent, which was shown to belong to Casals' group B arthropod-borne viruses and to be related to Russian spring-summer encephalitis (RSSE) virus, was called Powassan virus. Viruses of the RSSE complex, which cause serious human illness characterized by central nervous system and hemorrhagic manifestations, had been found previously only in Europe and Asia. During May 1952, 100 *Dermacentor andersoni* were collected along the North Cache la Poudre River, Colorado. A virus was isolated at the Rocky Mountain Laboratory from a pool of 20 of these ticks. The agent proved not to be Colorado tick fever (CTF) virus. Recently it has been shown to be closely related to Powassan virus. This paper reports data concerning isolation and serological characterization of this agent.

Materials and methods. In this laboratory isolations of viral agents from arthropods are carried out routinely according to the method of Eklund *et al.*(2). Antigens are prepared by extraction of suspensions of brains of infected suckling mice with acetone-ether according to the method of Clarke and Casals (3). Sera were prepared in adult mice by intraperitoneal (IP) inoculation on days 0 and 2 of 0.2 ml formalin-treated vaccine. Such vaccines consisted of a 10% suspension of brain tissue of infected suckling mice in 0.85% saline with formaldehyde added to give a final concentration of 0.5% formalin. On days 10, 15, 20, and 50 the animals were given 0.2 ml of a 10% suspension of infected brain by the same route. Mice were exsanguinated 7 to 10 days following last injection, and the serum obtained was designated as hyper-hyper-immune (HHI). HHI hamster serum was prepared by subcutaneous (SC) inoculation of adult hamsters with 0.03 ml of a 10^{-2} suspension of brains of infected suckling mice. Two additional SC inoculations of a 10^{-1} suspension of the same material were administered at 2-week intervals. The hamsters were bled 2 weeks after last inoculation. Hemagglutination (HA) and hemagglutination-inhibition (HAI) tests were done by the technics of Clarke and Casals(3), using erythrocytes from <1-day-old chicks. Sera were treated with kaolin to remove nonspecific inhibitors. For complement-fixation (CF) tests, all sera were inactivated at 60°C for 20 minutes. The next to highest dilution of complement showing complete hemolysis was used in the CF tests as 2 exact units. Antigen-serum mixtures were incubated overnight at 4°C before the hemolytic system was added. Virus neutralizations were performed with equal amounts of standard HHI mouse sera and serial 10-fold dilutions of virus incubated 1 hour in a 37°C water bath. Four-day-old Swiss albino mice (RML strain) were inoculated intracerebrally (IC) with 0.02 ml of this material and 21-day-old mice with 0.03 ml; by the IP route it was 0.05 ml for mice of either age. The Reed-Muench method was used to calculate LD_{50}(4).

Results. Isolation. The pool of 20 ticks, designated as No. 791, was triturated in 2 ml of 10% filtered normal rabbit serum in saline (FNRS-S). Each of a litter of six 4-day-old mice was inoculated IP with 0.05 ml of the supernatant of this suspension. For 7 days no signs were observed, and 3 of the mice were sacrificed. A suspension of their brains was inoculated IP into another litter. On the 8th day, one of the 3 remaining mice of the original litter was sick and on the 9th day, 2 were dead and one was sick. The last mouse died on the 13th day. On the 5th day, mice of the blind passage were sick, and in subsequent passages, incubation period has remained at 5 days. The isolate from this blind passage is designated as 791A-52 virus. Reisolation of virus from the original tick suspension was not possible, probably because the suspension had been left at room temperature for 1 day. When this agent was isolated, the only viruses being handled in the laboratory were those of CTF, yellow fever (YF) and St. Louis encephalitis (SLE). The isolate was shown not to be any of these 3 viruses. Since this virus had not been encountered previously in the laboratory, the isolation was considered authentic.

Serological characterization. Hemagglutination. Optimal conditions for demonstrating HA were pH 6.8 and 22°C. However, HA occurred at a pH range of 6.0 to 7.0 and at temperatures of 4°C, 22°C and 37°C. A rough HA pattern was exhibited at 4°C and pH 6.0; under all other conditions, a smooth HA pattern was observed.

Antigens prepared in the same manner from RSSE (Absettarov strain) and Powassan viruses were also tested for HA properties (Ta-

TABLE I. Hemagglutination Titers of Antigens Prepared from Brain Tissue of Suckling Mice Infected with Viruses of 791A-52, RSSE (Absettarov Strain) or Powassan.

Antigen	Temp., °C	pH 6.0	6.2	6.4	6.5	6.6	6.8	7.0	7.2
791A-52	4	640*	10240>†	10240>		10240>	10240>	5120	
	22	1280	5120	"		"	"	10240>	
	37	"	640	640		320	640	640	
RSSE (Absettarov)	4	0	0	0		"	"	160	
	22	0	0	0		0	0	0	
	37	0	0	0		0	0	0	
Powassan	4			0	1280	1280	0	0	0
	22			0	0	0	0	0	0
	37			0	0	0	0	0	0

* Signifies the reciprocal of highest dilution of antigen agglutinating < 1-day-old chick erythrocytes.

† > = or greater.

0 signifies hemagglutination titer <20.

RSSE and Powassan antigens exhibited rough agglutination; 791A-52, smooth agglutination.

ble I). A prozone was observed in the HA pattern produced by Powassan virus: hemagglutination did not occur in antigen dilutions of 1:20 and 1:40.

Hemagglutination-inhibition. Eight units of hemagglutinating antigen prepared from 791A-52 were tested against serial dilutions of HHI mouse sera prepared against Chikungunya, Eastern equine encephalitis, Sindbis and Western equine encephalitis viruses of group A (Casals' classification of arthropod-borne viruses), 10 viruses belonging to Group B (Table II), Bunyamwera and Wyeomyia viruses of the Bunyamwera group, miscellaneous viruses including CTF (Florio strain), lymphocytic choriomeningitis, Turlock and the mouse virus GD-7, as well as a strain of virus isolated in this laboratory from *Aedes trivittatus* and one from *Culiseta inornata*. HHI mouse sera prepared against 3, as yet unidentified, viruses, and HHI hamster sera prepared against 791A-52 virus were included in the HAI test. None of the sera except those prepared against viruses belonging to group B and the homologous virus inhibited agglutination. Serum dilutions which inhibited 8 HA units of antigen varied from 1:10 to 1:2560.

TABLE II. Inhibition of 791A-52 Hemagglutinin by Hyper-hyper-immune Mouse Serum Prepared against Various Group B Viruses.

Sera	Titer	Sera	Titer
Jap B (K-29)	2560*	Powassan	"
West Nile	"	St. Louis (Mosq. 798-55)	160
Murray Valley encephalitis	1280	Dengue (N. Guinea B)	"
Ilheus	"	Dengue (Hawaii)	10
St. Louis (Parton)	640	791A-52 (HHI hamster)	320
Zika	"		
Ntaya	320		

* Reciprocal of dilution of serum inhibiting 8 units of hemagglutinin.

Lowest dilution serum tested: 1/10.

Complement-fixation. Serial dilutions of 791A-52 antigen were tested against serial dilutions of HHI mouse sera prepared against a number of group B arthropod-borne viruses. HHI hamster serum prepared against the virus of 791A-52 was used in this study. Other group B viral antigens were tested against their homologous HHI sera as controls to demonstrate virus relationships. All of the homologous antigens were tested against other group B antisera (Table III). The antiserum prepared against Powassan virus was the only one which gave a high CF titer in presence of 791A-52 antigen. Antisera prepared against SLE (Parton strain), Japanese B (Korea-29 strain), Ilheus, Murray Valley encephalitis (MVE), and West Nile viruses showed only slight relationships to 791A-52 when tested against its antigen. Complement was not fixed when 791A-52 antigen was tested against antisera prepared against the viruses of Uganda-S, Zika, Ntaya, YF (17-D strain), or against serum obtained from normal mice.

Neutralization. The 791A-52 virus was neutralized by Powassan and RSSE HHI sera to titers approximately as high as those obtained with the homologous serum (Table IV). The 791A-52 HHI hamster serum neutralized Powassan and the homologous virus to about the same titer. The RSSE virus was neutralized by 791A-52 serum to low titer only in 21-day-old mice by the IP route. In

TABLE III. C-F Tests with Group B Viral Antigens and Their Respective Hyper-hyper-immune Mouse Sera.

Antigen	Sera 791A-52*	Powassan	St. Louis (Parton)	Jap B (K-29)	Ilheus	MVE	West Nile	a. Uganda-S b. Zika c. Ntaya d. YF (17-D) e. Normal
791A-52		32/128†	8/8	8/2	4/2	8/2	4/2	All 0/0
Homologous	128/256>‡	64/256>	128/256>	128/128	256/256>	256/256>	256/256>	a. 128/256> b. " c. 128/64 d. 64/64 e. 0/0

* Hyper-hyper-immune hamster serum—slightly anticomplementary.

† Numerator represents maximum dilution of serum fixing complement in presence of antigen; denominator, maximum dilution of antigen fixing complement in presence of antiserum. 0/0 signifies that no fixation occurred even when a 1:4 dilution of serum was mixed with the undiluted antigen.

‡ > = or greater.

TABLE IV. Cross-Neutralization Test (791A-52, Powassan and RSSE). LD_{50}'s and logs protection in mice.

		Sera															
		FNRS				791A-52*				Powassan				RSSE			
		4-day		21-day		4-day		21-day		4-day		21-day		4-day		21-day	
Virus		IC	IP	IC	IP	IC	IP	IC	IP	IC	IP	IC	IP	IC	IP	IC	IP
791A-52	T†	8.9	8.9>‡	8.4	7.5	6.2	4.2‖	5.7	1.5<§	4.8	2.3	4.7	1.5<	5.7	6.3>	5.7	2.5‖
	LP†					2.7	4.7>	2.7	6.0>	4.1	6.6>	3.7	6.0>	3.2	2.6±	2.7	5.0
Powassan	T	9.6	8.5	9.0	8.0	6.6	6.0	6.4	4.1‖	4.8	1.6<	5.0	1.5<				
	LP					3.0	2.5	2.6	3.9	4.8	6.9>	4.0	6.5>				
RSSE	T	9.3>	7.5	"	8.7	8.3>	6.5>	8.4>	5.9					6.4	3.6	6.3	1.8<
	LP					1.0<	1.0±	.6±	2.8					2.9>	3.9	2.7	6.9>

* Hyper-hyper-immune hamster serum diluted 3 times.

† T = titer. LP = logs protection.

‡ > = or greater.

§ < = or less.

‖ Prolonged incubation period and irregular death pattern.

another experiment (unpublished data), the RSSE serum did not neutralize Powassan virus by the IC route of inoculation in 21-day-old mice. Those data indicate that the relationship between 791A-52 and Powassan viruses and RSSE virus is nonreciprocal.

Conclusions. A virus closely related, if not identical, to Powassan virus has been isolated from *Dermacentor andersoni* from northern Colorado. A nonreciprocal relationship between RSSE virus and 791A-52 virus is indicated by the cross neutralization tests. The importance of *Dermacentor andersoni* in maintenance of this virus is unknown. Although thousands of this tick species collected in many areas in the western United States have been examined for viruses in this laboratory, this is the only isolation of this agent. Since these prior studies of ticks at the Rocky Mountain Laboratory were directed specifically toward study of the ecology of Colorado tick fever virus, the ecology of the 791A-52 virus is unknown. Field and laboratory studies on this subject are in progress.

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Since this paper was submitted, an article has appeared by J. Casals (*Can. Med. Assn. J.*, 1960, v82, 355) showing that Powassan virus is more closely related to the RSSE complex of viruses than to any other arbor virus and is a distinct virus.

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Connective Tissue III. Collagen and Hexosamine Content of Tissues of Rats of Different Ages.* (25837)

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Sobel and his co-workers have reported that hexosamine and collagen content of skin of rat, guinea pig, squab, rabbit and human, femur of rat and lung of rabbit(1,2,3,4) increase with age. They have also shown that rate of hexosamine deposition decreased with age more than that of collagen deposition; therefore, ratio of hexosamine to collagen decreased with age. Houck and Jacob(5) reported that concentration of hexosamine in rat skin was relatively unaffected by age of the animal, but ratio of hexosamine to nitrogen decreased with increases in body weight up to 330 g. Above this figure, however, the ratio varied directly with body weight. The present study is concerned with determination of the relation of age to: (1) total protein, (2) collagen and (3) hexosamine in aorta, tendon and skin of rat.

Methods. Male and female rats, Wistar strain, were used in this study. Rats from 4 weeks to 8 months of age were raised in our laboratory on Purina chow and water *ad lib.* Two-and-one-half-year-old rats were purchased from Research Supply Co., Philadelphia, Pa. One-tenth to 0.5 g of tissue were extracted with fat solvents(6). For total protein and collagen determinations the fat free tissues were hydrolyzed in 6N HCl at 110°C for 16 hours in a sealed tube. Nitrogen was determined by Conway microdiffusion method (7) and protein was calculated as 6.25 x nitrogen. Hydroxyproline was determined by the method of Neuman and Logan(8). Collagen in tendon and skin was calculated by multiplying the amount of hydroxyproline by

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7.46(9). Since rat aorta contains a constant level of 6% elastin(10) and rat elastin contains 2.3% hydroxyproline(9), amount of collagen in rat aorta was calculated as [total hydroxyproline—(6 x 2.3%)] x 7.46. For hexosamine determination, the fat-free tissues

TABLE I. Influence of Age on Content of Protein, Collagen and Hexosamine in Tissues of Rat (g/100 g of Tissue).

Tissue	Age	Protein	Collagen	Hexosamine	H/C ($\times 10^{-3}$)
Aorta*	4 wk (6)	22.8	7.4	.18	24.3
	A	B <.01	B <.01 CD <.001 E <.05	B <.01 E <.001	
	8 wk (4)	28.9	9.5	.23	24.2
	B	C <.01 E <.001			
	4-5 mo (6)	23.0	10.4	.21	20.2
	C			E <.02	
	8 mo (7)	25.1	10.4	.20	19.3
	D	E <.05		E <.01	
	2½ yr (4)	21.0	9.0	.25	27.8
	E				
	Pooled S.D.	± 2.6	± 1.2	± .03	
Tendon	4 wk (10)	32.2	23.1	.17	7.49
	A	B <.01 CD <.001 E <.02	BCDE <.001	C <.001 D <.02	
	8 wk (11)	38.1	30.2	.18	5.92
	B			C <.001 D <.01 E <.05	
	4-5 mo (15)	41.7	32.7	.13	3.95
	C				
	8 mo (10)	40.5	31.7	.14	4.21
	D				
	2½ yr (12)	37.9	30.4	.15	4.95
	E				
	Pooled S.D.	± 4.8	± 3.8	± .03	
Skin	4 wk (15)	24.2	12.9	.14	10.97
	A	C <.01 D <.001 E <.05	BCDE <.001	C <.02 E <.01	
	8 wk (10)	27.4	16.9	.16	9.36
	B		D <.001 E <.01	CE <.01 D <.02	
	4-5 mo (14)	27.3	19.4	.12	6.48
	C		D <.05		
	8 mo (12)	30.1	22.5	.13	5.60
	D				
	2½ yr (12)	26.9	21.4	.12	5.86
	E				
	Pooled S.D.	± 3.4	± 3.2	± .02	

Figures in parentheses represent No. of animals and corresponding No. of determinations unless otherwise specified.

P value relating to age differences, when within 5% level of confidence, is listed beneath results of actual determination and represents comparison between that figure and that of age groups designated by letter or letters preceding the value.

* Each determination consists of a pooled sample from 2-3 rats.

were hydrolyzed in 4N HCl in sealed tubes at 100° for 5 hours. Hexosamine content of the hydrolysate was determined by the method of Rondle and Morgan(11).

Results. Changes in the contents of protein, collagen and hexosamine in tissues of rat with age are shown in Table I. In *aorta* the protein content increased between 4 weeks and 8 weeks, then decreased in older animals. Collagen increased between 4 and 8 weeks of age with no significant change thereafter. Hexosamine increased between 4 weeks and 2½ years of age. There was no constant regular effect of age upon H/C ratio. In *tendon* the content of protein and collagen increased between 4 and 8 weeks and maintained high levels thereafter. Content of hexosamine decreased between 4 and 8 weeks and 4-5 months of age. H/C ratio decreased between 4 weeks and 4-5 months of age. In *skin* the content of protein increased between 4 weeks and 4-5 months and was relatively constant thereafter. Content of collagen increased between 4 weeks and 8 months of age. Hexosamine content was constant between 4-8 weeks, then decreased at 4-5 months. There was a decrease of H/C ratio with age between 4 weeks and 4-5 months.

Discussion. These data show a gradual increase in collagen content of skin from 4 weeks up to 8 months of age, while in the aorta and tendon the increment occurs between 4th and 8th weeks. Hexosamine content of aorta increases with age as compared to tendon and skin in which there is a decrease. Therefore, no change in H/C ratio was observed in aorta. The decreased H/C ratios in tendon and skin are the net result of increases in collagen and decreases in hexosamine with age.

Summary. 1. The relation of age to collagen and hexosamine content of aorta, tendon and skin was studied in rats from 4 weeks to 2½ years of age. Collagen content increased with age between 4 and 8 weeks in aorta and tendon; between 4 weeks and 8 months in skin. Hexosamine increased with age in aorta and decreased with age in tendon and skin.

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